Heterogeneity of clonal B cells in Multiple Myeloma

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1. Het verschijnen van oligoklonale serum immunoglobulines in multiple myeloma patiënten na autologe stamcel transplantatie moet niet beschouwd worden als vroege merker voor terugkeer van de ziekte, maar is een weerspiegeling van de uitgroei van normale B cellen, en daarmee dus een gunstige prognostische indicator (dit proefschrift, Zent et al. *Blood* 1998, 91(9) 3518-23).

2. Gezien het feit dat circulerende myeloma klonotypische B cellen verminderd in staat zijn om uit te rijpen tot volledig mature plasmacellen is het onwaarschijnlijk dat deze cellen ten grondslag liggen aan de relaps van de ziekte na intensieve behandeling (dit proefschrift).

3. Myeloma plasmacellen onderscheiden zich van normale plasmacellen door een significant lagere expressie van het CD27 molekuul. Expressie van CD27 op plasmacellen in myeloma patiënten na intensieve behandeling kan gezien worden als een gunstige prognostische factor (dit proefschrift).

4. Stimulatie van het CD27 molekuul op myeloma plasmacellen heeft een apoptotisch effect, terwijl het op plasmacellen van primaire plasmacel-leukemie patiënten een anti-apoptotisch effect heeft (dit proefschrift).

5. Primaire plasmacel-leukemie dient beschouwd te worden als een separate ziekte-entiteit op basis van immunofenotypering, genetische afwijkingen en klinisch beloop.

6. Multiple myeloma is een verzamelnaam voor verschillende op elkaar lijkende ziekten. Nieuwe technieken zoals mRNA microarray maken differentiële diagnose mogelijk en dragen bij aan verbeterde behandelstrategieën voor deze verschillende entiteiten.

7. Het succes van het geneesmiddel thalidomide in de behandeling van multiple myeloma patiënten is een typisch geval van farmaceutische serendipiteit.

8. In de behandeling van multiple myeloma patiënten dient het 'toevoegen van leven aan de jaren' in plaats van het 'toevoegen van jaren aan het leven' het primaire doel te zijn.

9. Weten wat je weet en weten wat je niet weet, dat is ware kennis. (Confusius 551-479 v. Chr. 'Bloemlezingen').

10. Aangezien bij mannen de bierconsumptie niet geassocieerd is met de 'body-mass-index' noch met de 'waist-hip-ratio', is het begrip 'bierbuik' onzinnig (Bobak et al. *Eur.J.Clin.Nutr.* 2003, 57(10) 1250-3).

11. Wetenschappers zijn beter in het uitvoeren van wetenschappelijke experimenten dan in de (maatschappelijke) extrapolatie van de vergaarde kennis.

12. De mogelijke gezondheidsrisico's van gen-mutaties zullen nauwkeurig bepaald moeten worden om tot een duidelijke definitie van de term 'ziekte' te komen in het huidige 'genomics' tijdperk.

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'He that will apply new remedies must expect new evils; for time is the greatest innovator.'

(Sir Francis Bacon 1561-1626)

Contents

chapter one

General introduction

MULTIPLE MYELOMA: HISTORICAL OVERVIEW

The Russian physician Dr. J. von Rustizky introduced the term 'multiple myeloma' in 1873, describing a patient in which he found eight tumour masses in the bone marrow during autopsy.[1] In 1889, the Austrian Dr. Otto Kahler described a patient suffering from bone pain and spontaneous fractures. Dr. Kahler recognised numerous large cells in the bone marrow and noticed that the urine of this patient contained the typical protein described by Dr. Bence Jones.[2] Earlier, in 1845, Mr. Thomas McBean consulted his physician Dr. Macintyre because of severe chest pains and fatigue. In 1846, Mr. McBean died; the official cause of death was listed as 'atrophy from albuminuria'.[3] A urine sample was sent to Dr. Henry Bence Jones who described the physical properties, he noticed that it showed a 'protein' precipitate when heated at 40-58°C after addition of a weak acid. Dr. Bence Jones showed much interest in the protein and recognised the importance of identifying this 'albumen oxide' in other cases of 'bone softening'.[4,5]

More than 100 years later, Edelman & Gally demonstrated that the light chains prepared from an immunoglobulin G (IgG) monoclonal protein were identical to the Bence Jones protein from the same patient's urine. They studied the heat properties of these light chains and showed that it was identical to the heat properties described by Bence Jones.[6] In 1928, Perlzweig and colleagues were the first to describe serum hyperproteinaemia in a myeloma patient.[7] The development of immunoelectrophoresis by Grabar & Williams in 1953[8] simplified the detection of monoclonal proteins in myeloma patients, which greatly facilitated the diagnosis of multiple myeloma. In 1968, Kunkel demonstrated that monoclonal myeloma proteins consisted of heavy and light chains, which could also be detected among normal immunoglobulins (Ig) and antibodies. He concluded that the monoclonal proteins were produced by malignant plasma cells and were structurally equal to antibodies produced by normal plasma cells.[9] Waldenström introduced the concept of monoclonal versus polyclonal gammopathies. He described patients presenting with a narrow immunoelectrophoresis spike but without obvious evidence of malignancy. He termed this condition 'benign monoclonal gammopathy'.[10] Currently, the term 'monoclonal gammopathy of undetermined significance' is more broadly used, because some of these patients eventually progress towards overt multiple myeloma or related disorders.[11] Marschalkó (1895) was the first to accurately describe plasma cell characteristics, including the eccentric position of the nucleus, a pale perinuclear hof area and a spherical or irregular cytoplasm.[12] In 1900 Wright described a patient from which he examined the bone marrow and concluded that the tumour consisted of plasma cells or the immediate descendants of these cells. Wright furthermore described plasma cells in normal bone marrow and stated that multiple myeloma was 'a neoplasm originating in only one of the varieties in the red marrow, the plasma cells'.[13,14]

Nowadays, the presence of malignant plasma cells and monoclonal Igs (paraprotein/M-component) detected in serum and urine (Bence Jones protein) are still the most important

Table I. Diagnostic criteria for Multiple Myeloma (after Durie and Salmon[15]).
The diagnosis of Multiple Myeloma requires a minimum of one major and one minor criterion, or three minor criteria, including bone marrow plasmacytosis (a).

Major criteria	Minor criteria
I. Plasmacytoma on tissue biopsy. II. Bone marrow with > 30% plasma cells. III. M-component in serum: IgG > 3 g/dL, IgA > 2 g/dL. Bence Jones protein in urine (k or λ) > 1 g/24 hours.	(a) 10-30% plasma cells in the bone marrow. (b) M-component: present but lower levels than mentioned in major criteria. (c) Lytic bone lesions. (d) Reduced normal immunoglobulins (<50% normal): IgG < 600 mg/dL, IgA < 100 mg/dL, IgM < 50mg/dL.
Indolent/smouldering Multiple Myeloma	
Same as Multiple Myeloma except: No bone lesions or one asymptomatic lytic lesion (X-ray survey). M-component present but at levels IgG < 3 g/dL, IgA < 2 g/dL, urine Bence Jones protein < 4 g/24 hours. Hemoglobin > 10 g/dL, serum calcium normal, serum creatinine < 2mg/dL, no infections.	
Monoclonal Gammopathy of Undetermined Significance	
M-component present, but at levels IgG < 3 g/dL, IgA < 2 g/dL, urine Bence Jones protein < 1 g/24 hours. Normal level of residual polyclonal immunoglobulins. Bone marrow plasma cells < 10% (without cytological abnormalities). No bone lesions and/or bone pain, normal physical examination. No symptoms or associated disease features. Hemoglobin > 10 g/dL, serum calcium normal, serum creatinine < 2 mg/dL, no infections.	

diagnostic criteria for multiple myeloma (Table I).[15] Other important criteria include anaemia, osteolytic lesions, and renal failure, which are mainly caused by Ig light chain deposition and hypercalcemia.

CLINICAL ASPECTS OF MULTIPLE MYELOMA

Multiple Myeloma (MM) is an incurable malignancy of plasma cells. The bone marrow is the most prominent localisation of the tumour, although myeloma plasma cells and clonotypic B cells can also be found in low numbers in the peripheral blood. MM accounts for approximately 1% of all cancers and about 10% of haematological malignancies and has an approximate incidence of 3.5 per 100,000 population. The median age of newly diagnosed MM is about 65 years. Although much effort has been made to improve overall survival in MM, the median survival is approximately 3 years. Clinical trials conducted during the past 40 years show only a modest improvement of survival. Combination chemotherapy consisting of alkylating drugs such as melphalan, vincristine and doxorubicine, in combination with dexamethasone or prednisone is the most applied treatment modality for MM patients. Response rates vary and an effective salvage therapy after failure of first-line therapy is still lacking.[16-19] Randomised controled clinical trials and meta-analysis of data from individual patients who participated in

clinical trials show that there is actually no difference in survival between patients receiving combination chemotherapy and patients receiving melphalan plus prednisone.[20,21] Interferon-α has been used as maintenance therapy in MM. However, the beneficial effects are limited. The progression-free survival improves with interferon, but the overall survival remains unaffected.[22] Recent studies point out that high-dose chemotherapy followed by reinfusion of autologous stem cells improves the response rate, the event-free survival and overall survival.[23,24] Purging strategies to remove myeloma clonal cells from the autograft have been pursued extensively. Positive selection for the stem cell marker CD34 reduces myeloma cells contamination in peripheral blood stem cell collections.[25-27] However, clinical studies show no improvement of overall survival of MM patients receiving purged autografts.[28,29] In order to maximise the complete response rate the double or 'tandem' transplant treatment regimen has been designed. The first clinical results show a longer complete response duration compared to single transplantation.[30,31] However, randomised trials are still ongoing and await final analysis. From clinical studies it can be concluded that the median survival for autologous stem cell transplantation is superior to allogeneic stem cell transplantation. Transplant-related mortality is significantly higher for patients receiving allogeneic stem cell transplants.[32] However, patients receiving an HLA matched stem cell transplant have a lower relapse-rate, indicative for a graft versus myeloma effect.[33,34] This strategy is now further explored in the setting of reduced intensity allogeneic stem cell transplantation.[35-37] Despite all efforts, treatment failure is still a major problem in management of MM. The appreciation of (increased) bone marrow angiogenesis in active disease prompted investigators to explore the use of anti-angiogenic drugs to treat MM patients. Recently, it has been demonstrated that the anti-angiogenic drug thalidomide is active in advanced MM patients, providing a therapeutic option for refractory MM.[38] A phase II clinical study demonstrated that refractory and smouldering MM patients treated with thalidomide have improved response rates.[39] Preliminary results indicate that thalidomide may also be effective in early-stage MM, delaying the progression towards symptomatic disease.[40] Currently, immunomodulatory derivatives of thalidomide are under investigation in phase I-II clinical trials.[41,42] Proteasome inhibitors represent a novel class of anticancer drugs. These agents inhibit the degradation of ubiquitinylated proteins, thereby downregulating anti-apoptotic pathways and cell cycle progression. It has been demonstrated that the proteasome inhibitor PS-341 induces apoptosis of MM cells and inhibits paracrine MM growth *in vitro*.[43] A phase I study showed that PS-341 is active in refractory MM.[44] A randomised controlled trial determining the efficacy of PS-341 with dexamethasone as salvage therapy is currently underway.

Although much progress is obtained with regard to survival prolongation, long term survival is still not achieved. The cellular and molecular basis for treatment-resistance and disease persistence remains poorly understood. Circulating B cells expressing identical immunoglobulin receptors as the myeloma plasma cells in the bone marrow have been identified using anti-

idiotype antibodies and sensitive PCR techniques based on the hypervariable regions of the V(D)J rearrangement expressed by the myeloma plasma cells. It has been hypothesised that these so-called clonotypic B cells are the myeloma precursor cells and reflect a chemotherapy-resistant pool of disease, which might be responsible for relapse.

MULTIPLE MYELOMA AND RELATED DISEASES

Monoclonal Gammopathy of Undetermined Significance (MGUS) is a pre-malignant disorder that affects 2% of persons over the age of 50 years. It is characterised by the presence of a monoclonal serum protein, but the patients have less than 10% plasma cells in the bone marrow. A large retrospective study by Kyle *et al.* demonstrated that 115 of the 1384 studied patients progressed to multiple myeloma or related disorders. The calculated risk of progression of MGUS is about 1% per year and the initial concentration of serum monoclonal protein was highly predictive of progression within 20 years.[45] Amyloidosis is related to MGUS. However, in this disorder the monoclonal protein deposits in various tissues leading to organ failure. The most common presentations of amyloidosis include renal insufficiency, hepatomegaly, heart failure and peripheral neuropathy.[46] Like multiple myeloma, these tumours are characterised by intramedullary growth. They differ from multiple myeloma regarding the number of plasma cells in the bone marrow (<10%). In smouldering myeloma the percentage of plasma cells in the bone marrow generally exceeds 10%, but patients lack the other typical myeloma related complications such as osteolytic lesions. Of the patients with multiple myeloma 5 to 15% can be classified as smouldering myeloma at presentation.[47] In some multiple myeloma patients the malignant plasma cells display extramedullary growth, mostly in peripheral blood, skin and pleural fluid. Extramedullary growth of plasma cells usually occurs upon progression of the disease and is denominated plasma cell leukaemia. Plasma cell leukaemia can be primary or secondary, depending on whether patients presented with preceding intramedullary growth. Primary or *de novo* plasma cell leukaemia accounts for approximately 1 to 2% of the multiple myeloma cases and has an aggressive disease history compared to intramedullary multiple myeloma.[48,49] It has been suggested that primary plasma cell leukaemia represents a separate clinical entity that can be distinguished from multiple myeloma on basis of immunophenotyping and chromosomal abnormalities.[50-55]

B CELL DIFFERENTIATION AND MYELOMA DEVELOPMENT

Plasma cells originate from B cells and reflect the end-differentiated stage of this cell population. B cell differentiation is characterised by distinct phenotypic and genetic alterations, eventually leading to the generation of antigen-specific B cells. B cells develop in the bone marrow, where precursor B cells firstly undergo Ig heavy chain (IgH) V(D)J rearrangement which is mediated by the recombination activating gene proteins RAG1 and RAG2.[56-59] Diversity is generated because the rearrangement is assembled from 38 to 46 functional Vh genes,

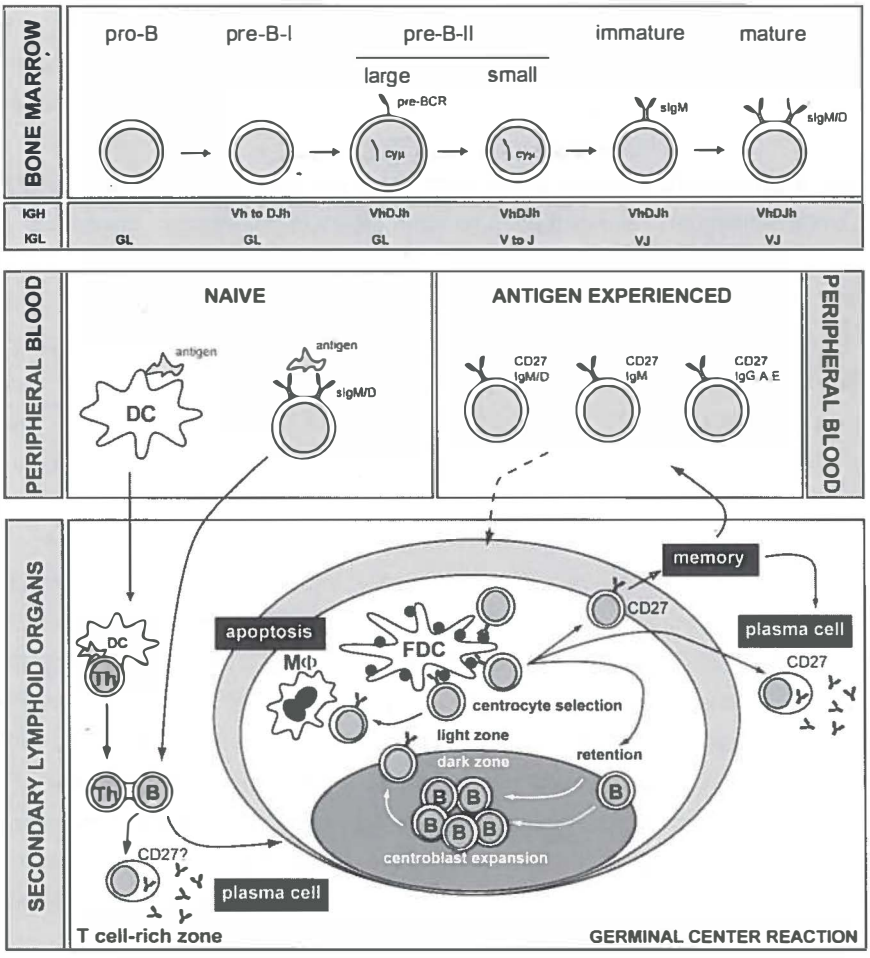

Figure 1. Human B cell differentiation. Upper panel: Human B cell development in the bone marrow, according to Ghia *et al.* [88] Middle and lower panel (partly adapted from McHeyzer-Williams [89]): Antigen-specific activation of circulating naive B cells. B cell differentiation taking place in the T cell-rich areas results in formation of short-lived plasma cells, it is unclear whether these short-lived plasma cells express CD27. In the germinal center antigen-activated B cells undergo clonal expansion and somatic hypermutations. Antigen-specific selection of high affinity B cells results either in differentiation into $CD27^+$ memory B cells, which re-enter the peripheral blood, or into $CD27^+$ long-lived plasma cells, which migrate to bone marrow.

grouped in 7 gene families, 27 D genes, grouped in 7 gene families and 6 Jh genes.[60-62] Initially, D to Jh rearrangement takes place, followed by Vh to D-Jh rearrangement.[62,63] The terminal deoxynucleotidyl transferase (TdT) protein is expressed at this stage and is involved in the random addition of nucleotides at the Vh-D-Jh junctions.[64] Successful rearrangement leads to surface expression of the μ heavy chain which is bound to the surrogate light chain encoded by the λ14.1 and VpreB genes.[65-67] On the cell surface this forms a complex with the Igα and Igβ signalling molecules.[65-69] B cell maturation beyond this point depends on adequate B cell receptor

signalling. Subsequently, Ig light chain rearrangement takes place, which is initiated at the κ light chain locus. Productive rearrangement downregulates the expression of the RAG proteins. When productive rearrangement fails, the other κ light chain allele is rearranged. Unsuccessful rearrangement on both κ alleles leads to rearrangement at the λ light chain locus.[70,71] Although in most pre-B cells rearrangements at the light chain loci take place in this strictly ordered fashion, λ rearrangements have also been found in cells with germline or disrupted κ loci.[70-74] Pairing of the rearranged light chain with the μ chain enables further differentiation into a surface IgM expressing B cell. At this stage, negative selection of autoreactive B cells takes place in the bone marrow. Autoreactive immature B cells become anergic, die by apoptosis or are induced to undergo secondary Ig light chain rearrangement (receptor editing).[75,76] Immature B cells that are not negatively selected migrate from the bone marrow to the peripheral blood, where differentiation into mature B cells takes place (Figure 1: upper panel).

Naive B cells in the peripheral blood are antigen-inexperienced. B cells are activated by antigen which crosslinks the surface immunoglobulin receptor, leading to internalisation and processing of the antigen (Figure 1: middle panel). The processed antigen is presented in the context of MHCII molecules. Antigen-primed B cells are recruited into the germinal center reaction that is initiated in the T cell-rich zones of secondary lymphoid organs. Here, antigen-specific T and B cell interactions take place. The presented antigen on the B cell is recognised by the T cell receptor on the surface of the T helper cell. This interaction is costimulated by the CD28-CD86 and the CD40-CD40L interactions, which are required for germinal center formation.[77,78] Next to the germinal center reaction, many activated B cells directly differentiate into plasma cells, which mainly express IgM and are considered to be predominantly short-lived.[79,80] Upon cognate interaction with primed T cells, rapid clonal expansion of B cells takes place resulting in the formation of a secondary follicle, which polarises into the typical dark and light zone of a germinal center. B cells in the dark zone are denominated centroblasts. They proliferate and undergo somatic hypermutation (SHM), resulting in a high rate of mutations into the variable regions of the Ig rearrangements. Centroblasts exit the cell cycle as centrocytes and migrate to the light zone of the germinal center. Antigen is presented in the light zone in the context of immune complexes on the surface of follicular dendritic cells. At this stage centrocytes are selected on basis of affinity for the presented antigen. Diminished antigen-affinity leads to apoptosis whereas improved antigen-binding results in positive selection. Positively selected B cells may re-enter the dark zone and undergo additional rounds of expansion, diversification and selection (Figure 1: lower panel). Centrocytes in the germinal center may also undergo Ig class switch recombination (CSR), by which the constant region of the Ig molecule, responsible for the effector function of the humoral immune response, is altered.[81-83] Thereby, IgM and IgD expressing centrocytes switch to expressing IgG, IgA or IgE. Ig CSR is mediated by intrachromosomal deletions between switch regions located upstream of the constant region genes, resulting in juxtaposition of rearranged V(D)J complex to downstream constant region

genes. Eventually, selected B cells may exit the germinal center and recirculate into the periphery as memory B cells, or migrate to the bone marrow and differentiate into long-lived plasma cells.[84,85] As a consequence of V(D)J rearrangement and the subsequent diversification process in the germinal center, post-germinal center B cells are equipped with highly diversified clonal B cell receptors. The rearrangement expressed by B cells can be regarded as a highly specific clonal marker that can be used to analyse and trace malignant B cells[86], and determine whether these are of pre- or post-germinal center origin.[87]

Multiple myeloma plasma cells most probably originate from long-lived plasma cells in the bone marrow. They have a very low proliferative capacity that is reflected by a DNA-labelling index of 1 to 2%. Molecular analysis of the expressed V(D)J rearrangements revealed extensive somatic hypermutations, which pattern suggests that the myeloma clone is antigen selected.[90,91] The absence of intraclonal mutation heterogeneity indicates that multiple myeloma is of post-germinal center origin.[92] Furthermore, the myeloma derived V(D)J rearrangement is observed from presentation to plateau phase of the disease, providing evidence that progression does not involve clonal evolution or a new B cell clone.[93] It has been proposed that multiple myeloma involves several oncogenic steps leading to transformation.[94,95]

The stability of the V(D)J rearrangement in multiple myeloma implies that crucial oncogenic events have taken place in a post-germinal center B cell. The majority of multiple myeloma cases have chromosomal translocations involving the Ig heavy chain locus at 14q32. Molecular analysis has demonstrated that most of these translocations involve the switch regions and are mediated by erroneous Ig CSR.[96-100] The almost uniformal presence of such switch translocations suggests that these are primary events in myelomagenesis. These data suggest that the germinal center plays a pivotal role in the development of multiple myeloma and myeloma clonotypic B cells. Whether the germinal center reaction is involved in genetic aberrations other than primary switch translocations is uncertain.

AIM OF THE THESIS

Despite many efforts, MM remains a disease that is difficult to treat and consequently has a poor prognosis. The apparent heterogeneity in treatment response eventuates in the appreciation that the term 'multiple myeloma' might very well describe a group of patients suffering from related symptoms caused by several different pathways. Deregulation of these pathways, and possible oncogenic consequences, are dependent on maturational status and cellular context in which they take place. The understanding of myeloma heterogeneity is closely associated with the ontogeny of the myeloma clone. Therefore, the aim of this thesis is to characterise the heterogeneity of the myeloma clone and its relationship with myeloma ontogeny. The presence and functional aspects of circulating myeloma clonotypic B cells, the proposed myeloma precursor cells, have been investigated, as well as immunophenotypic heterogeneity of myeloma plasma cells and possible implications on prognosis.

In **chapter 2** an overview is given of the current knowledge regarding heterogeneity in myeloma plasma cell morphology, immunophenotype, chromosomal abnormalities and other genetic alterations, as well as the role of clonotypic B cells, immunoglobulin isotype switch variants and molecular profiling. The consequences of this heterogeneity on prognosis and clinical course are discussed.

In **chapters 3 and 4** the phenomenon of oligoclonal serum Igs in MM patients was investigated. The persistence and clinical significance of oligoclonal serum Igs were studied by follow-up of 37 MM patients. We questioned whether persistent oligoclonal serum Igs are related to outgrowth of clonotypic B cells expressing variants Ig-isotypes, and thus can be regarded as a marker of relapse.

In **chapter 5** functional aspects of circulating clonotypic B cells (expressing variant Ig-isotypes) were studied. The *in vitro* proliferation and differentiation capacities of clonotypic B cells were studied using the CD40-culturing system, analysing the presence and relative number of clonotypic B cells under different culture conditions.

Based on the presence of somatic mutations in the V(D)J rearrangements expressed by clonotypic B cells these cells are considered to be of memory B cell origin. The recent identification of CD27 as a memory B cell specific surface marker prompted us to study the expression of CD27 in MM patients. In **chapter 6** the expression CD27 was evaluated by flow-cytometry in a cross-sectional MM patient group. Expression of CD27 mRNA in purified plasma cells from 74 newly diagnosed MM patients was studied by cDNA microarray. Results were associated with clinical data and known prognostic factors.

Finally, in **chapter 7** the functional consequences of CD27-triggering on plasma cells from *de novo* plasma cell leukaemia was studied, especially in relation to dexamethasone-induced apoptosis.

In **chapter 8 a** summary of the results described in this thesis is given. The results and future research perspectives are discussed.

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chapter two

Heterogeneity in the multiple myeloma tumor clone

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SUMMARY

Multiple Myeloma (MM) is a plasma cell malignancy which is characterized by a very heterogeneous disease outcome. Heterogeneity in plasma cell characteristics, including morphology, maturation status, immunophenotype and genetic abnormalities partly account for the variable disease outcome. Although the plasma cell is the predominant cell type in MM, several studies have shown that less mature B cells, which are clonally related to the malignant plasma cells, are present in the bone marrow and peripheral blood of MM patients. The significance of these so-called myeloma clonotypic B cells in the disease process remains largely unknown. In this review the role of myeloma clonotypic B cells and myeloma tumor clone heterogeneity in relation to prognosis and clinical outcome are discussed.

MYELOMA PLASMA CELL MORPHOLOGY

The presence of plasma cells in the bone marrow is still the most important diagnostic feature for MM. In healthy individuals plasma cells account for about 1-2% of the cells in the bone marrow, in MM plasma cells typically exceeds 10% of all bone marrow cells. Morphological diagnosis of MM is relatively easy, bone marrow sections and smears show increased numbers of plasma cells. In May-Grünwald-Giemsa or Wright-Giemsa stainings of bone marrow, typical plasma cells cells are morphologically characterized by abundant blue cytoplasm, pale perinuclear zone (hof) and eccentric nucleus with coarse chromatin. Plasma cells may contain several inclusions, which can be large, cytoplasmic and eosinophilic (Russel bodies) or nuclear (Dutcher bodies). Plasma cells can also be increased in non-malignant pathological conditions; so-called reactive plasmacytosis. Morphological distinction between reactive and malignant plasma cells can be very difficult. In contrast to malignant plasma cells, plasma cells in reactive plasmacytosis are mostly mature, do not show nucleocytoplasmic asynchrony, rarely appear in clusters and usually have no nucleoli. Normal human plasma cells from tonsil, peripheral blood and bone marrow display different morphological and phenotypic characteristics. A gradient of increasing maturity from tonsil to blood to bone marrow can be observed.[2] Immature plasma cells show a denser nucleus and less cytoplasm and are more observed in tonsil and peripheral blood. Although myeloma plasma cells in most cases closely resemble normal mature bone marrow plasma cells, abberant morphological features of myeloma plasma cells have been described for incidental cases. Myeloma plasma cells may present with multiple nuclei, which can appear multilobated, convoluted, lobular and vacuolated. The low incidence of MM patients presenting with plasma cells displaying abberant morphology impairs the determination of a prognostic value for these phenomena. Stratification of MM patients according to ultrastructural abnormalities including the presence of cytoplasmic and nuclear inclusion bodies and dense body appearance did not show significant differences in survival.[3] However, plasmablastic plasma cell morphology has been recognized as a prognostic factor for many years in MM. Several studies reported on this subset of MM patients (10-20%) presenting with plasmablastic plasma cells, which have a more aggressive clinical course.[4-8] Plasmablastic plasma cells are characterized by a fine reticular chromatin pattern in the nucleus (no or minimal chromatin clumping); a large nucleus, or a large nucleolus; the cytoplasm has no or very little hof region, and less abundant cytoplasm (less than one-half of the nuclear area). Studying a large cohort of MM patients, the prognostic value of plasmablastic morphology has recently been confirmed. Greipp *et al.* showed that MM patients presenting with immature (plasmablastic) plasma cells have a higher degree of adverse prognostic factors.[9] Studying a group of 453 MM patients, they found 38 patients (8.2%) with a plasmablastic morphology. These cases had significant higher bð2-microglobulin and serum calcium levels, a higher percentage of bone marrow plasma cells and higher plasma cell labeling indices showing that plasmablastic MM cells have a higher proliferation rate compared to typical plasma cells. Furthermore, when treated with

combination chemotherapy, MM patients with plasmablastic morphology showed a significant lower response rate than non-plasmablastic MM. The progression rate of plasmablastic MM was also higher, and event-free and overall survival was shorter. Multivariate analysis showed that plasmablastic morphology is an independent prognostic factor. Recently, it has been shown that plasmablastic morphology is also an independent prognostic factor for poor survival in relapsed and refractory MM patients undergoing autologous stem cell transplantation.[10] Based on these results Goasguen *et al.* designed a morphological algorithm capable of distinguishing four cellular subtypes which are related to clinical outcome. Immaturity of plasma cells was related to a poor outcome, again indicative for the heterogeneity of plasma cell morphology and its clinical consequences.[11] To date, phenotypic, molecular and cytogenetic characteristics of the plasmablastic MM are limited. In future, these data might provide a better understanding of the biology of this subgroup of MM patients and new tools to classify and recognize plasmablastic MM.

IMMUNOGLOBULIN ISOTYPE SWITCH VARIANTS

The analysis of the immunoglobulin variable (VH and VL) and constant (CH) genes expressed by myeloma plasma cells has provided important insights into the B cell maturational status in which the final transforming event has taken place. Myeloma plasma cells express identical immunoglobulin heavy and light chain rearrangements coding for the monoclonal immunoglobulin which can be detected in serum and urine of patients (paraprotein / M-component). These rearrangements carry a high load of somatic mutations without intraclonal variation. Furthermore this rearrangement is stable throughout the course of the disease. The pattern of somatic hypermutations suggests that the myeloma tumor clone has undergone antigenic selection in a germinal center reaction. The absence of intraclonal variation provides evidence that clonal expansion has taken place in a post-germinal center B cell.[12,13] Typically, myeloma plasma cells secrete a monoclonal immunoglobulin (Ig) which is mostly of post-switched isotype (IgG or IgA). In a subgroup of patients (~20%) only Ig light chains are expressed (Bence-Jones type disease). IgA expressing MM and Bence-Jones type MM patients have a poorer prognosis compared to IgG expressing MM patients, which is probably due to the higher incidence of renal failure in these patients.[14,15]

A small group of patients (1-5%) presents with non-secretory MM. Patients presenting with myeloma plasma cells expressing pre-switch immunoglobulins (IgM or IgD) are very rare (IgD ~2%; IgM 0.2%)[16] and the issue whether these cases can be classified as typical MM or as Waldenström's macroglobulinemia is still under debate. Pre-switch immunoglobulin MM cases are characterized by a plasmablastic/lymphoplasmacytic morphology and have a lower immunoglobulin-secreting ability, compatible with an immature plasma cell differentiation stage. IgM secreting MM cases frequently present with clinical symptoms which are characteristic for lymphomas like splenomegaly and hepatomegaly, and generally do not have osteolytic

lesions[17] [own unpublished observation]. Regarding morphology and clinical features, IgD-expressing MM is not significantly different from classical switched MM.[17] Furthermore, sequence analysis revealed that pre-switch MM express somatically mutated VH-genes without intraclonal variation.[18,19] IgD expressing MM cases display a high number of somatic mutations and a Cμ-Cδ switch recombination. The normal IgD expressing counterpart is present in human tonsil and carries a comparable number of mutations and also harbours the Cμ-Cδ switch junction.[20] Strikingly, IgD MM cases have a predominant expression of lambda light chains (>60% of the cases).[21,22] It is unclear why IgD MM displays such a light chain shift. It has been speculated that secondary light chain rearrangements (receptor editing) are involved in the bias towards lambda expression.[20] However, using Southern blotting and DNA sequencing, van der Burg *et al.* have found no evidence that IgD MM cases had expressed another Ig light chain prior to the in-frame lambda light chain, providing evidence that receptor revision is not involved in biased lambda expression.[23]

The low incidence of IgM MM hampers evaluation of prognosis. IgD MM is generally associated with a poor prognosis and an aggressive clinical course, although well-documented patient studies are still lacking. Recently, it has been shown that MM patients expressing atypical immunoglobulin isotypes (IgM, IgE and non-secretory MM) are characterized by a higher incidence of t(11;14) chromosomal breakpoint, involving the *CCND1* locus at 11q13 and the *IGH* locus at 14q32.[17]

Not only the expressed immunoglobulin isotype, but especially the serum and/or urine concentration of the expressed paraprotein has prognostic value in MM, as this is a reflection of the plasma cell load in patients. Paraprotein concentration is an important parameter to monitor disease activity and response to therapy. The Durie and Salmon criteria[24] are most commonly used for the staging of MM patients, whereby the paraprotein concentration is an important criterium in this staging system and concomitantly an important prognostic factor in MM.

Following high-dose chemotherapy and autologous stem cell transplantation a high incidence of multiple immunoglobulin isotypes in the serum was reported.[25-27] The appearance of different isotypes after treatment which differ from the original isotype at presentation imposes a problem for the management of treatment. Post-treatment oligoclonality could be due to isotype switching of the myeloma tumor clone, the emergence of a new malignant clone different from the initial clone, or abberant B cell development (clonal dysregulation). The serum oligoclonality is transient in most cases. In a follow-up study by Zent *et al.* it was demonstrated that patients with detectable oligoclonality had significant higher complete response to therapy and a better overall and event-free survival compared to patients without post-treatment oligoclonality.[25] Using a sensitive PCR assay based on the hypervariable part of the immunoglobulin heavy chain rearrangement expressed by the myeloma plasma cells, we demonstrated that the post-treatment serum oligoclonality is not caused by immunoglobulin

switching of the malignant clone but rather reflects the dysclonal recovery of normal B cell development.[26] Consequently, post-treatment oligoclonality can be regarded as a marker for the reconstitution of the normal immune function in MM patients.

B cells expressing indentical VDJ rearrangements as the malignant plasma cells but coupled to different immunoglobulin constant regions have been detected by us and other groups using PCR-based methods using oligonucleotides specific for the hypervariable CDR1, CDR2 and CDR3 regions combined with Cμ, Cγ and Cα specific oligonucleotides. Importantly, clonotypic pre-switched B cells were detected in bone marrow and peripheral blood of IgG and IgA secreting MM patients.[28] The clonotypic variant isotypes have been detected in the $CD38^{-}$ $CD45^{+}$ bone marrow fraction, but not in the plasma cell fraction.[29] These data suggest that the clonotypic cell in MM can originate from a pre-switched, somatically mutated B cell, which is consistent with the model of a more immature precursor cell in MM. Interestingly, clonotypic VDJ-transcript of post-switch isotypes (Cγ2 and Cγ3) have also been detected in IgM-secreting MM, whereas such isotype variants were not detected in Waldenströms macroglobulinemia.[30] Thus, IgM-secreting MM likely originates from a memory B cell which is undergoing isotype switching and is arrested in the differentiation stage just preceding that of typical isotype-switched MM.

The relative low success-rate of detecting clonotypic cells expressing variant isotypes suggests that these cells are present at low abundance. The malignant potential and clinical significance of myeloma clonotypic cells expressing variant isotypes remains elusive. To adress this issue, Reiman *et al.* determined the presence of clonotypic isotype variants in patients undergoing therapy using various PCR strategies.[31] At presentation they found clonotypic isotype variants in the bone marrow in 22 of 26 patients using nested PCR. Clonotypic IgM transcripts were detected in 18 out of 26 patients by nested-PCR. Furthermore, they detected multiple clonotypic isotypes in the peripheral blood obtained after stem cell mobilization in 7 out of 17 patients. Clonotypic IgM transcripts were only detected by nested PCR in 2 out of 17 patients. In contrast to our findings[26], Reiman *et al.* were able to detect clonotypic isotype variants after high-dose chemotherapy followed by autologous stem cell reinfusion. Clonotypic IgM transcripts were detected in 4 out of 17 patients before treatment which persisted after auto-transplantation. In 3 out of 17 patients clonotypic IgM transcripts newly appeared after stem cell reinfusion. Clonotypic isotype variants can only be detected by RT-PCR, which hampers the evaluation of the frequency of such cells. Therefore, Reiman *et al.* used the percentage of blood samples containing clonotypic IgM transcripts as a relative measure for the load of clonotypic IgM cells in MM patients. Patients were divided into a low IgM-detection rate (<50% of evaluated samples) and a high IgM-detection rate (>50%) group, and it was shown that low IgM-detection rate patients have a significantly better overall survival than high IgM-detection rate patients. Furthermore, it was shown that IgM-detection rate correlated with other known prognostic factors (e.g. β2-microglobulin, % bone marrow plasma cells). It must be emphasized that the IgM-detection rate is a relative measure which is highly dependent on factors like quality of

the samples, efficiency of RNA-isolation and the difference of sample number for each patient, and should therefore be used with caution. The relation between clinical outcome and clonotypic IgM suggests that these cells are involved in disease activity. However, it remains to be defined whether such cells have intrinsic malignant potential. They might reflect a MM progenitor cell which undergoes directed isotype-switching and continuous differentiation into plasma cells. Alternatively, it might be that these cells are long-lived clonal memory B cells which persist in the peripheral blood and bone marrow but in which the final neoplastic event has not taken place. Lastly, it might be that pre-switch and post-switch isotypes are expressed by the same cells, which may be the result of some form of abberant switch recombination (e.g. trans-switching, alternative trans-splicing). Using an *in vitro* culturing system capable of sustaining B cell growth we demonstrated that clonotypic isotype variants are present among peripheral blood B cells, and already expressed at the circulating B cell differentiation stage. Furthermore, clonotypic isotype variants could be subcloned *in vitro*, arguing against the hypothesis that multiple isotypes are expressed by the same cell.[32]

Generally, malignant cells may be able to engraft in immunodeficient mice. Reiman *et al.* demonstrated the persistence of clonotypic cells variant isotypes *in vivo* by xenografting bone marrow cells from MM patients in NOD/SCID mice.[31] These characteristics might be indicative for the malignant potential of these cells, although these transcripts could only be found by nested PCR, illustrating that clonal expansion of these cells does not appear to take place *in vivo*. Additionally, clonotypic isotype variants have been studied in the murine 5T myeloma model. Bakkus *et al.* found clonotypic isotype variants in the bone marrow of the IgG expressing 5T2 and 5T33 bearing mice. Interestingly, no isotype variants were detected in the *in vitro* variant of 5T33. However, when injected into syngeneic mice, isotype variants could be found in the bone marrow of 5T33-injected animals. In this system, the appearance of clonotypic IgM transcripts could only be generated by trans-switching as the Cμ region was lost from the cis-chromosome.[33] Consequently, this suggests that clonotypic pre-switch cells can not be regarded as a myeloma precursor population but is the result of abberant switching activity within the malignant clone. Therefore, the detection of these isotype variants might be indicative for disease activity, but not informative about the origin of the myeloma tumor clone.

CIRCULATING CLONOTYPIC B CELLS

Plasma cells are the predominant cell type in MM, and primarily involved in the clinical characteristics of MM. However, myeloma plasma cells have a modest proliferative capacity, and specifically reside and home to the bone marrow. Consequently, myeloma plasma cells are rarely detected in peripheral blood and circulating plasma cells are only readily detectable in progressive end-stage disease patients (secondary plasma cell leukemia). These features are not in line with the expected growth and spreading characteristics of a malignant precursor

cell. In early studies peripheral blood (monoclonal) B cells bearing the malignant plasma cell idiotype were detected by using anti-idiotype antibodies[34-36]. These findings were confirmed in later studies using the hypervariable immunoglobulin rearrangement expressed by the malignant plasma cells as a target in the polymerase chain reaction. Such allele-specific oligonucleotide (ASO) PCR approaches identified clonotypic cells among the circulating $CD45^+$ / $CD38^-$ / $CD19^+$, and even $CD34^+$ cell populations. These findings led to the hypothesis that circulating clonotypic B-lineage cells might be regarded as the proliferative pool and responsible for the dissemination of the disease. Furthermore, the observation that clonotypic B cells in MM functionally express the multidrug transporter molecule P-gp170 (MDR1) suggests that clonotypic B cells represent a drug resistant cell population responsible for relapse of the disease after treatment.[37,38] The frequency and clinical significance of circulating clonotypic B cells is a disputed issue. Pilarski *et al.* reported that peripheral blood B cells expressing clonotypic VDJ rearrangements constitute a large subset. Using single-cell and in-situ PCR they found that the majority (>60%) of peripheral blood B cells are clonotypic.[39] In contrast, Chen *et al.* reported that peripheral blood B cells of MM patients comprise only a small population of clonotypic cells (<6%).[40] Furthermore, it has been suggested that the total number of B cells is elevated in the peripheral blood of MM patients, and that these cells express CD34 on their surface. Again, the majority of these $CD34^+$ circulating B cells were reported to express clonotypic IgH rearrangements.[41] In addition, they observed that the majority of clonotypic $CD34^+$ / $CD19^+$ circulating B cells display DNA hyperdiploidy, which is characteristic for malignant cells,[42] and express IL-6 and IL-6 receptors, the major growth/survival factor for MM cells.[43] Other studies, however, could not confirm these findings. In these studies clonotypic cells were not detectable in $CD34^+$ cell populations, demonstrating that the detection of clonotypic transcripts was due to $CD34^+$ sorting procedure.[44-46] Studying the number of circulating T and B cells in the blood of large cohorts of MM patients, Kay *et al.* showed that $CD19^+$ blood B cell levels were not significantly elevated in MM patients compared to the blood B cell levels in age-matched normal controls. ASO-PCR analysis revealed no significant correlation between the number of circulating B cells and the number of clonotypic B cells.[47] Furthermore, elevated $CD19^+$ peripheral blood B cells were not associated with poor prognosis but were positively associated with survival of the patients, which argues against the malignant potential of these cells.[48]

Analysis of structural and numerical chromosomal abnormalities in clonotypic B cells has been studied only very limited. These studies are hampered by the low frequency of these cells. Davies *et al.* did not find numerical chromosomal abnormalities in $CD20^+$ peripheral blood cells, while Zandecki *et al.* detected numerical abnormalities in a small number of $CD20^+$ B cells in only 2/15 patients.[49,50] Zojer *et al.* detected cytogenetically abberant cells (gain chromosome 11, deletion of RB-1, deletion of p53) in a small fraction of the CD19-sorted peripheral blood cell population of 6 MM patients using FISH in combination with

immunocytochemical analysis of Ig light chain expression.[51] For one patient, we showed that highly purified $CD19^+$ $CD138^-$ peripheral blood B cells harboured indentical t(4;14)-specific fusion-transcripts as the malignant plasma cells in the bone marrow and that these transcripts were still readily detected after *in vitro* B cell stimulation.[32] These studies suggest that only a small fraction of clonotypic B cells are cytogenetically abnormal and therefore closely related to the malignant plasma cells. Whether the final oncogenic event has already taken place in these cells is not clear. Consequently, conclusions regarding the malignant status of clonotypic B cells can not be made from these studies.

The presence and frequency of clonotypic B cells in patients undergoing high-dose chemotherapy and autologous stem cell or allogeneic stem cell transplantation could provide important insights into the clinical importance of these cells and is therefore the subject of intense study. Several groups have assessed the frequency of clonotypic B cells in MM patients undergoing treatment. Bergsagel *et al.* showed that $CD19^+$ clonotypic B cells could be detected in peripheral blood by PCR irrespective of chemotherapeutic treatment.[52] Similar results were obtained by Kiel *et al.*, Rottenburger *et al.* and Rasmussen *et al.* who demonstrated that clonotypic cells persist in the $CD19^+$ and $CD20^+$ cell fraction after high-dose chemotherapy.[53-55] However, the number of persisting clonotypic cells in these studies was low in accordance with the study performed by Chen *et al.* Concommitantly, the presence of clonotypic B cells was analyzed in mobilized peripheral blood stem cells used for autografting MM patients. Purging strategies to remove residual clonotypic B cells from the autograft mostly involved positive selection of $CD34^+$ cells. This selection procedure generally reduces the number of clonotypic cells in the autograft with 2 to 5 logs. Despite purging methods, clonotypic B cells were still detectable in autografts.[26,44,56] Similar results were obtained using a negative selection for B cell markers like CD19, CD20, CD22 and CD37.[57]

The myelomagenic potential of clonotypic B cells present in autografts was tested in an *in vivo* setting by injecting (unselected) mobilized blood from MM patients into irradiated NOD/SCID mice. Pilarski *et al.* showed that some inoculated mice develop MM-like disease symptoms including osteolytic lesions and human immunoglobulins in the serum. Furthermore, these features were transferable to secondary bone marrow recipients. Pilarski *et al.* concluded that clonotypic B cells have the ability to generate myeloma in this model and thus can be regarded as the myeloma precursor population.[58] However, from these experiments it cannot be excluded that residual circulating plasma cells and not clonotypic B cells are responsible for the observed phenomena. Whether clonotypic B cells present in autografts are instrumental in the reappearance of the malignant clone remains elusive. More stringent purging strategies are needed to remove all contaminating clonotypic B cells from autografts and to determine their clinical significance.

IMMUNOPHENOTYPIC HETEROGENEITY

Phenotypically, myeloma plasma cells closely resemble normal mature plasma cells. However, myeloma plasma cells can be distinguished from normal plasma cells on basis of the expression of several surface markers (Table I). The asynchronous expression of these markers can thus be regarded as tumor-specific characteristics. Furthermore, the myeloma clone apparently not only includes mature plasma cells but also immature plasma cells, which are characterized by the expression of B cell lineage markers. Important phenotypic characteristics of plasma cells which are routinely used in the diagnosis and management of the disease are the expression of CD138 (Syndecan-1),[59] the strong expression of CD38, and the absence or dim expression of CD45. CD138 is a heparan sulphate bearing proteoglycan and is involved in cellular adhesion, and the regulation of the activity of heparin-binding cytokines. CD138 can also be shed from the membrane of myeloma cells; this soluble CD138 inhibits the *in vitro* proliferation of myeloma plasma cells.[60] The serum level of shed CD138 is an independent prognostic factor,[61] probably because it is strongly associated with the tumor load. High expression of CD38 is commonly used as a plasma cell marker. CD38 is also expressed by other cell lineages (albeit at a lower intensity) and therefore less suitable for single-parameter analysis of plasma cells. In flow-cytometry settings, CD38 staining in combination with CD45 or cytoplasmic light chain staining provides a robust plasma cell phenotyping strategy. Moreover, the combination of CD45 and CD38 allows not only the indentification of mature plasma cells which are characterized by the $CD38^{++}/CD45^{-}$ phenotype but also more immature $CD38^{++}/CD45^{+}$ plasma cells. Schneider *et al*. showed that $CD45^{+}$ plasma cells in MM patients display an immature plasma cell morphology and consisted of either poly- or monoclonal plasma cells in contrast to the $CD45^{-}$ plasma cell population which invariably were monoclonal.[62] Furthermore, it has been demonstrated that proliferating myeloma cells are predominantly found in the $CD45^{+}$ immature plasma cell fraction. $CD45^{+}$ plasma cells responded strongly to IL-6 and are more sensitive to undergo apoptosis. IL-6 stimulation of $CD45^{-}$ plasma cells reinduced CD45 expression which is again lost after IL-6 withdrawal.[63,64] These studies suggests that immature myeloma cells represent the proliferating, IL-6 responsive population. In addition, it has been shown that myeloma plasma cells express higher levels of the IL-6 receptor α-chain (CD126) compared to normal plasma cells in the bone marrow.[65] $CD45^{+}$ and $CD45^{-}$ plasma cells have also been identified in the 5T mouse model for human myeloma. Interestingly, $CD45^{+}$ plasma cells had a lower proliferative capacity compared to $CD45^{-}$ plasma cells. After injection of sorted $CD45^{+}$ or $CD45^{-}$ into syngeneic mice both $CD45^{+}$ aswell as $CD45^{-}$ plasma cells could be detected upon development of the disease, indicating that in this model $CD45^{-}$ plasma cells can differentiate into $CD45^{+}$ plasma cells and vice versa.[66]

Immature myeloma plasma cells have also been shown to express CD10. By ASO-PCR it has been demonstrated that the CD10-positive subset harbours clonal cells.[67,68]

Myeloma plasma cells can be distinguished from normal plasma cells on basis of the expression

of CD56 and the lack of CD19 expression. CD56 (NCAM) is a natural killer cell antigen. Van Camp *et al.* reported that approximately 78% of MM patients present with $CD56^+$ plasma cells. In that study, CD56-expressing plasma cells were not found in MGUS patients and normal donors. Furthermore, $CD56^-$ MM patients had a higher incidence of extramedullary disease, a higher β2-microglobulin. $CD56^-$ plasma cells were more frequently associated with plasmablastic morphology.[69] Additionally, it has been reported that $CD56^-$ MM patients have a significantly lower overall survival.[70] However, other studies could not confirm these clinical data and report the expression of CD56 in MGUS patients.[71,72] Several studies demonstrated that CD56 is downregulated in extramedullary MM and *de novo* plasma cell leukemia,[73-75] suggesting that CD56 expression on myeloma plasma cells is involved bone marrow retainment and downregulation of CD56 in extravasation of myeloma plasma cells.

Expression of CD19 is specifically lost from myeloma plasma cells, whereas it is expressed on plasma cells in the bone marrow of normal donors.[76] Enforced expression of CD19 on myeloma plasma cells inhibits *in vitro* and *in vivo* growth, suggesting that $CD19^-$ plasma cells in MM have a growth advantage and thus might be clonally selected.[77] The combination of plasma cell CD56 and CD19 staining was tested in a flow cytometry setting to evaluate its applicability as monitor of disease and efficacy of treatment in MM. San Miguel *et al.* have intensively studied the abberant phenotype of myeloma plasma cells and have shown that the $CD56^+$ $CD19^-$ criterium is suitable to evaluate tumor load in patients. They furthermore demonstrated that patients who have >30% phenotypically normal plasma cells had a significantly longer event-free survival.[78] In the study by Rawstron *et al.* a similar approach lead to comparable results. Additionally, they showed that $CD56^+$ $CD19^-$ myeloma plasma cells were detectable in patients who achieved a complete remission and were negative in the immunofixation, illustrating the sensitivity of this assay.[79] However, $CD56^-$ MM cases apparently obscure the results using this approach. An exceptional MM case was recently identified displaying $CD19^+$ $CD56^-$ phenotype.[80]

Another molecule involved in myeloma plasma cell adhesion is CD44. Compared to normal plasma cells myeloma plasma cells show overexpression of the CD44v9 variant isoform, which mediates binding to the bone marrow stromal cells and is involved in the induction of IL-6 by bone marrow stromal cells. Expression of CD44v9 on myeloma plasma cells is associated with poor prognosis.[81-84]

The mature B cell associated marker CD20 is expressed on myeloma plasma cells in approximately 20% of the MM patients and in 50% of plasma cell leukemia patients. It has been suggested that CD20-expressing myeloma reflects a more aggressive MM subtype, as $CD20^+$ MM cases have a shorter survival than $CD20^-$ cases.[85] In MM patients, CD20 is expressed in a heterogeneous fashion on plasma cells.[86] Furthermore, Interferon-γ (IFN-γ) potently induces and upregulates CD20 expression.[87] These findings provided the rationale to determine the applicability of CD20-directed therapy in MM patients using the anti-CD20 chimeric antibody rituximab, which has recently been shown to be effective in the treatment of non-Hodgkin

Table I. Immunophenotypic characteristics of myeloma plasma cells.

marker	name	normal distribution	expression in MM	function	function in MM	ligand	references
CD10	CALLA	B and T precursors, germinal center B cells	aberrant	Zinc-binding metalloprotease, regulates B cell development.	GC-derived CD10+ MM precursor population? Aggressive MM subtype.	-	Caligaris-Cappio et al. JCI vol.76, 1985 Cao et al. Leukemia vol.9, 1995 Kurabayashi et al. Cancer Res. vol.48, 1988
CD19	84, Leu12	B cells, follicular dendritic cells	lower/absent	B cell receptor complex, B cell activation.	Expressed on clonotypic B cells. Lost on MM PCs. Growth inhibition of MM PCs.	-	Harada et al. Blood vol.81, 1993 Mahmoud et al. vol.94, 1999
CD20	B1, Bp35	B cells	aberrant	Ca2+ channel subunit.	Unknown, expressed on clonotypic B cells. Aggressive MM subtype. Possible target for immunotherapy.	-	Rottenburger et al. Br.J.Haematol. vol.106, 1999 San Miguel et al. Br.J.Haematol. vol.77, 1991 Treon et al. J.Immunotherap. vol.25, 2002
CD27	TAPA-1	T cells, NK cells, memory B cells, plasma cells	lower/absent	T and B cell costimulation, plasma cell differentiation of memory B cells. .Apoptosis?	Unknown, lost from MM PCs.	CD70	Guikema et al. Br.J.Haematol. vol. , 2003 Katayama et al. Br.J. Haematol. vol. , 2003
CD28	Tp44	T cells	aberrant	T cell costimulation.	Associated with tumoral expansion. Involved in endogeneous interleukin-B production.	CD80 CD86	Pellat-Deceunynck et al. Blood vol.84, 1994 Robillard et al. Clin.Cancer Res. vol.4, 1998 Shapiro et al. Blood vol.98, 2001
CD40	Bp50	Mature B cells, follicular dendritic cells, activated monocytes	aberrant	B cell costimulation. Germinal center reaction. Isotype switching.	Involved in autocrine/paracrine IL-6 secretion. Proliferation.	CD154	Westendorf et al. J.immunol. vol.152, 1994 Teoh et al. Blood vol.95, 2000
CD44v9	Pgp-1	Hematopoietic and non-hematopoietic cells	overexpress-ed	Stroma adherence, extracellular matrix binding. Homotypic adhesion.	BM homing. Poor prognostic factor.	ECM hyaluronan	Stauder et al. Blood vol. 88, 1996 van Driel et al. Blood vol.12, 1998 Eisterer et al. Leuk.Res. vol.25, 2001
CD45	LCA	Hematopoietic cells except erythrocytes	immature and circulating MM PCs	T and B cell activation.	Unknown, expressed on proliferating MM PC population.	-	Schneider et al. Br.J.Haematol. vol97, 1997 Mahmoud et al. Blood vol.92, 1998 Ishikawa et al. Leuk.Lymphoma vol.39, 2000
CD56	NCAM	NK cells, T cells, neuronal cells	aberrant	adhesion.	BM homing and adhesion. Inversely correlated with number of circulating MM PCs.	CD56 ECM	van Camp et al. Blood vol.76, 1990 Pellat-Deceunynck et al. Leukemia vol. 12, 1998 Rawstron et al. Br.J.Haematol. vol.104, 1999
CD117	c-kit	Hematopoietic stem cells, mast cells	aberrant	hematopoietic stem cell development.	Proliferation/survival of MM PCs.	c-kit ligand (SCF)	Ocqueteau et al. 1996 Lemoli et al. 1996
CD126	IL-6R	B cells, plasma cells	overexpress-ion	B cell growth factor.	Proliferation/survival of MM PCs.	IL-6	Rawstron et al. Blood vol. 96, 2000

lymphomas.[88-90] Preliminary results show that rituximab might also be effective for MM patients, especially in combination with IFN-γ treatment.[87] Furthermore, circulating clonotypic B cells in MM also express CD20 and may therefore be an important additional target for rituximab. Another marker which is normally lost from plasma cells but is retained on MM plasma cells is CD40. Crosslinking of CD40 on MM plasma cells by its natural ligand CD154 resulted in increased autocrine and paracrine IL-6 secretion by bone marrow stromal cells and thereby enhanced MM plasma cell survival and proliferation.[91-93]

CD117 (c-kit) has been reported to be expressed on myeloma plasma cells in 32% of the patients but was absent on normal bone marrow plasma cells.[94] However, CD117 is also expressed on plasma cells from MGUS patients.[95] It is unclear whether these $CD117^+$ plasma cells can be considered as malignant plasma cells. Interaction of CD117 with c-kit ligand might be involved in growth/survival of MM cells.[96] In mast cell leukemia, activating mutations in the intracellular domain of c-kit have been found, redendering ligand-independency.[97] However, no such mutations have been detected in MM.

Another important myeloma-specific marker is CD28. Pellat-Deceunynck *et al.* reported that CD28 is expressed in the majority of the MM patients but is lacking on normal plasma cells from tonsil and bone marrow.[98] Studying 116 MM cases Robillard *et al.* demonstrated that CD28 is expressed in 19% of the MGUS patients, 41% of the MM patients, and 100% of 13 human MM cell lines. Furthermore, they showed that CD28 is expressed in 93% of the extramedullary relapse (secondary plasma cell leukemia) confirming the results with the MM cell lines which were all derived from the peripheral blood of MM patients with extramedullary relapse.[99] The function of CD28 expressed on myeloma plasma cells has been studied by Shapiro *et al.* who showed that CD28 stimulates transcriptional activity involving the CD28 Responsive Element and Nuclear Factor IL-2B AP-1 (RE/AP) sites. This contains both a Nuclear Factor-κB (NF-κB) and an Activator Protein-1 (AP-1) binding site and thereby increases IL-8 production. It has been proposed that IL-8 production by myeloma cells is involved in promoting angiogenesis.[100]

Recently, we and others demonstrated that myeloma plasma cells show loss of CD27 expression in contrast to normal bone marrow plasma.[101,102] Studying a cross-sectional patient group we showed that CD27 expression is expressed heterogeneously in MM, in which CD27 expression was significantly higher in patients who achieved a complete clinical remission compared to newly diagnosed and relapsed patients. In many MM patients at diagnosis we found $CD27^+$ aswell as $CD27^-$ plasma cells. ASO-PCR analysis on sorted populations showed that both populations can belong to the malignant clone. It is unclear whether $CD27^-$ plasma cells are derived from clonal $CD27^+$ plasma cells or whether both populations are derived from separate (clonal) precursor populations (Figure 1). CD27 expression on myeloma plasma cells was significantly correlated with CD19 expression as determined by mean fluorescence intensity analysis, suggesting that expression of CD27 is associated with plasma cell immaturity. CD27 was absent on nine MM human cell lines, providing evidence that loss of CD27 is associated

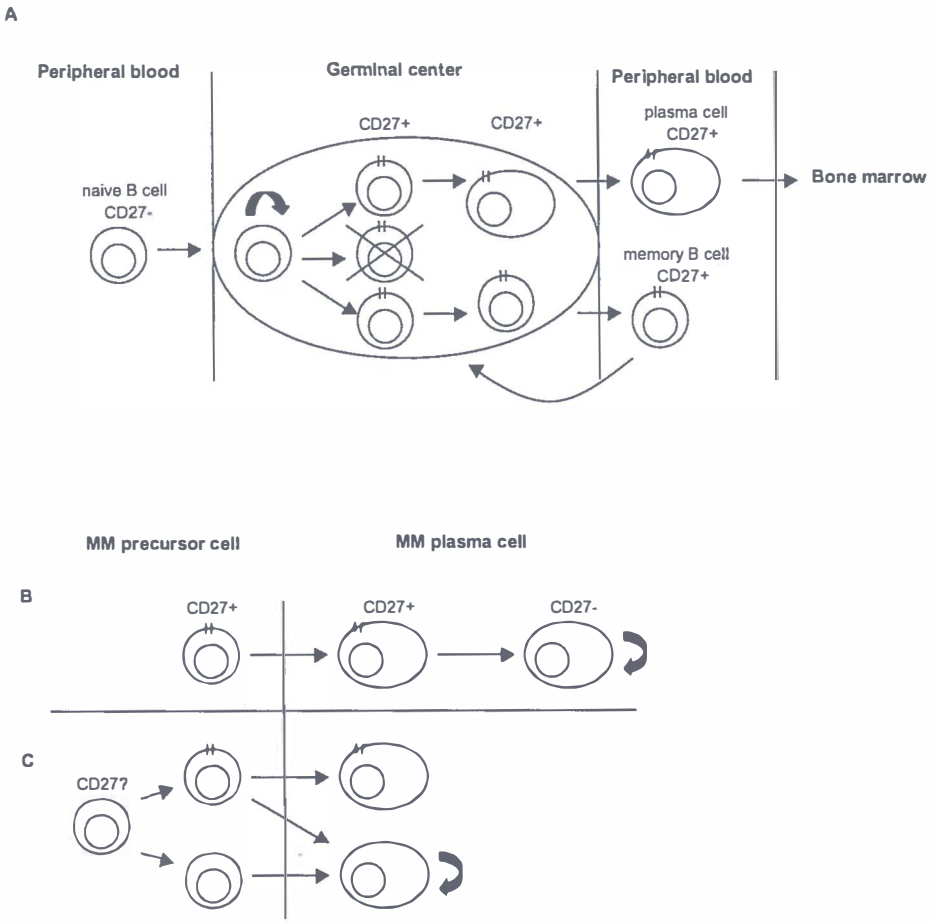

Figure 1: Development of normal and malignant plasma cells. A). Normal plasma cells development. Upon activation, $CD27^-$ naive B cells migrate from the peripheral blood to the dark zone of germinal centers. B cells proliferate, acquire somatic mutations in the VDJ genes in the darkzone of germinal centers and subsequently pass through the light zone. Expression of CD27 is acquired at this stage. Germinal center B cells expressing high affinity immunoglobulins are selected and differentiate into $CD27^+$ memory B cells or $CD27^+$ plasma cells. Memory B cells and plasma cells migrate to the peripheral blood. Upon reactivation, memory B cells can re-enter the germinal center and undergo a second round of selection. Plasma cells migrate to the bone marrow and differentiate into Ig-secreting cells. B). Proposed model for $CD27^+$ and $CD27^-$ plasma cells in multiple myeloma. $CD27^+$ MM precursor cells differentiate into $CD27^+$ plasma cells which subsequently differentiate into $CD27^-$ plasma cells. During progression $CD27^-$ show increased proliferation and gain precursor cell independency. C). Alternative model for $CD27^+$ and $CD27^-$ plasma cells in multiple myeloma. Clonal $CD27^+$ and $CD27^-$ precursor cells originate from common precursor and give rise to $CD27^+$ and $CD27^-$ plasma cells. During progression, $CD27^-$ plasma cells show higher proliferative potential and outgrow $CD27^+$ plasma cells.

with progressive disease. We suggest that during progression of disease myeloma plasma cells become increasingly independent from its precursor populations, this might explain why loss of CD27 coincides with progression.

In contrast, plasma cells from MGUS patients displayed a homogeneous high CD27 expression. Strikingly, CD27 was also highly expressed in *de novo* plasma cell leukemia (PCL) despite its typical aggressive clinical behaviour, which might suggest that *de novo* PCL is a distinct disease entity different from MM. The differential expression of CD27 on MM plasma cells was confirmed by cDNA microarray analysis. Comparing the mRNA expression of more than 5,000 genes, CD27 was the second most downregulated gene in MM plasma cells compared to normal donor bone marrow plasma cells.[103] Importantly, CD27 was differentially expressed in newly diagnosed MM patients, CD27 expression was lowest in the hierarchical clustered subgroup closely resembling human MM cell lines which was characterized by the highest prevalence of adverse prognostic factors. In contrast, CD27 expression was highest in the subgroup closely resembling normal plasma cells.[101]

The exact role of CD27 and loss of CD27 expression in MM remains to be determined. Katayama *et al.* studied the consequences of CD27 ligation by co-culturing CD27-transfected human MM cell lines (U266 and KMS-5) with a CD70-transfected cell line (NIH3T3). By yeast-two hybrid screening, the proapoptotic protein Siva was previously reported to bind to the cytoplasmic tail of CD27.[104] Upon CD27-ligation on transfected MM cell lines it was shown by immunoprecipitation that Siva was not associated with CD27 in MM. Concommitantly, CD27-ligation on transfected cell lines did not induce apoptosis. Furthermore, cDNA microarray analysis revealed a significant upregulation of the ectodermal neural cortex 1 gene (*ENC1*), but the relevance of this observation is still unclear.[102] In *de* novo plasma cell leukemia we showed that CD27-ligation inhibited dexamethasone-induced apoptosis which was associated with enhanced AP-1 activity (Guikema *et al.* in press). Currently, we are determining whether CD27 expression on plasma cells in MM patients has prognostic value as a clinical parameter.

In summary, the immunophenotype of myeloma plasma cells is not a fixed feature but displays marked heterogeneity and plasticity. To date, it is relatively easily to distinguish myeloma plasma cells immunophenotypically from normal cells, which contributes to the diagnosis and management of the disease. Immunophenotypical heterogeneity within the myeloma tumor clone should be carefully assessed to determine whether the used markers are suitable. The immunophenotype of myeloma cells may be affected by chemotherapeutic treatment and consequent clonal selection. The heterogeneity of the immunophenotype is associated with the function of the affected differentially expressed proteins and therefore provide useful targets to study their role in the malignant process. Furthermore, they provide potential new targets which may be used in new (immuno)therapeutic strategies.

HETEROGENEITY IN CHROMOSOMAL ABNORMALITIES AND GENETIC ALTERATIONS

Conventional karyotyping is difficult in MM due to the limited proliferative capacity of myeloma plasma cells. Therefore, karyotypic abnormalities are detected in only 30-50% of the MM patients and displays a high degree of heterogeneity. As yet, no MM specific genetic lesion has been identified. Detection of karyotypic abnormalities correlates with the stage of the disease which can be accounted to a higher proliferation rate of myeloma plasma cells in progressive disease. The use of more sensitive techniques (Fluorescence *In Situ* Hybridization (FISH) and Comparative Genomic Hybridization (CGH)) identified complex numerical and other unbalanced chromosomal abnormalities in nearly all primary MM cases and in all human MM cell lines.[105-109] Recurrent numerical abnormalities are trisomies of chromosome 3, 5, 7, 9, 11, 15, 19 and monosomy 13. Deletions at 13q and monosomy 13 are almost always monoallelic and are present in approximately 50% of MM patients as assessed by a two-probe interphase FISH approach. Deletions 13q14 are associated with a poor prognosis. Patients with 13q14 deletions have higher β2-microglobulin serum levels, higher degree of bone marrow plasmacytosis, higher paraprotein serum levels, higher incidence of lambda light chain expression and increased myeloma plasma cell proliferation.[110,111] In combination with β2-microglobulin serum levels, deletion 13q is a very strong prognostic factor in MM.[112] MM patients with chromosome 13 abnormalities detected by classical cytogenetics have a significant shorter event free and overall survival than patients with chromosome abnormalities detected by FISH.[113] Recently, using a multiprobe FISH approach Shaughnessy *et al.* showed that 86% of the MM patients harbour deletions at 13q, suggesting that the deletion of a specific chromosomal region at 13q is involved in the adverse prognosis.[114] Elnenaei *et al.* identified a minimal common deleted region of approximately 350 kb using a contig of large probes (yeast-, P1- and bacterial-artificial chromosomes) spanning 13q14-q21 in an interphase FISH strategy studying 82 MM cases. *RPF2* is a putative tumor suppressor gene which is located in the vicinity of the minimal common deletion region. The functional *RPF2* allele displayed no mutations in MM, suggesting that it may be silenced in a different manner.[115] The retinoblastoma gene (Rb-1) mapping to 13q14 is a tumor suppressor gene.[116] However, monoallelic deletions of Rb-1 in MM were shown not to alter the expression of the rb-protein[117], suggesting that an alternative mechanism may be involved in the inactivation of the Rb-protein. It has been shown that IL-6 signaling can inactivate the Rb-protein by means of phosphorylation, and thereby promote myeloma plasma cell proliferation.[118] Additionally, deletions of 13q14 were also detected in a fraction of patients suffering from MGUS. Strikingly, MM patients with a reported MGUS history had a higher incidence of deletion 13q compared to MM patients without a previous MGUS history, suggesting that deletion 13q might be involved in the transition of MGUS to overt MM, but is less important in the pathogenesis of 'de novo' MM.[119] Long-term follow-up of 18 MGUS patients showed that 5 patients harboured 13q14 deletions, which all progressed to overt MM, supporting the notion that deletion 13q14 is involved in the MGUS to MM transition.[120]

Recurrent reciprocal (balanced) chromosomal translocations have been found in MM (reviewed by Bergsagel & Kuehl and Fenton *et al.*[121-123]). These translocations mostly involve the immunoglobulin heavy chain (IgH) locus at 14q32 and various partner chromosomes. The ocurrence of such IgH translocations in MGUS patients suggests that they occur early in the pathogenesis of MM. The IgH translocations almost invariably involve the switch regions which rearrange during isotype class switch recombination (CSR) in the germinal center. This suggests that these IgH switch translocations are mediated by erroneous CSR in mature B cells undergoing a germinal center reaction. Recurrent partner loci in IgH switch translocations are: 11q13 (*CCND1*; approximate incidence 15-20%),[124] 4p16 (*FGFR3/MMSET*, approximate incidence 15%),[124-126] 6p21 (*CCND3*; approximate incidence 5%),[127] 6p25 (*MUM1/IRF4*; approximate incidence 5%),[128] 8q24 (*C-MYC*, approximate incidence 1%),[129,130] 16q23 (*C-MAF*, approximate incidence 5-10%)[131] and 20q11 (*MAFB*; approximate incidence 5%).[132,133] The remaining 40-50% involves various non-recurrent loci. Studies into the prognostic value of the recurrent chromosomal translocations involving the IgH locus are still limited. The t(4;14) and t(14;16) chromosomal translocations correlate with 13q deletions and these patients are characterized by a poor prognosis, probably imposed by the 13q deletions.[134] Strong associations between recurrent chromosomal translocations and patient survival as described for deletion 13q have not been identified. Interestingly, the t(11;14) was found to be associated with plasmablastic morphology. However, in a large patient study Fonseca *et al.* showed that these patients do not have a worsened prognosis.[135] IgM, IgE and non-secretory MM cases display a striking high incidence of the t(11;14), which was associated with plasmablastic morphology in especially IgM-secreting MM.[17] The t(11;14) was never found in Waldenströms macroglobulinemia or in lymphoma cases expressing switched Ig isotypes and displaying plasmablastic morphology. The t(11;14) has also been reported in light-chain amyloidosis.[136] These data suggest that the t(11;14) can be generally considered as a very early genetic event. The better prognosis which has been described for MM cases with t(11;14) could primarily be accounted to the better survival of patients with non-secretory MM.

The presence of chromosomal translocations involving the IgH switch regions in MGUS and light chain amyloidosis suggest that they reflect primary early events in MM. If clonotypic B cells are to play a role in the pathogenesis of MM we hypothesize that these chromosomal translocations result from defective class switch recombination in clonotypic B cells. This also implies that the CSR process is continued on the non-translocated IgH allele, yielding a class-switched malignant clone. Of special interest is the correlation between Ig-isotype, morphology and chromosomal translocations, showing that heterogeneity in the MM tumor clone is extended at the genetic and molecular level. Additional evidence that MM is a heterogeneous entity comprising different maturational stages comes from cDNA microarray gene expression studies.

MOLECULAR PROFILING OF MM PATIENTS

Molecular profiling using cDNA microarray has proven to be a powerful tool to distinguish subgroups among patients which may be considered homogeneous regarding histological, morphological, immunophenotypic and even genetic criteria.[137] This technique has the potential to identify pathways involved in pathogenesis and tumorigenesis. Furthermore, the consequences of chromosomal abnormalities on gene expression can be carefully assessed. Studies including morphological, immunophenotypic and genetic analysis clearly indicate that MM is a heterogeneous disease entity, therefore cDNA microarray analysis provides an attractive strategy to stratify MM patients according to gene expression profiling. Studying purified plasma cells from 74 untreated MM patients Zhan *et al*. showed by cDNA microarray profiling and hierarchical clustering that 4 distinct MM subgroups (MM1, MM2, MM3, MM4) could be identified.[103] The gene expression pattern of the MM1 subgroup closely resembled that of purified bone marrow plasma cells from healthy indivuals and MGUS patients, whereas the MM4 subgroup resembled MM cell lines. Comparing patient subgroups MM1 and MM4, most upregulated genes in MM4 were involved in cell cycle control and DNA repair, suggestive for a more proliferative phenotype. Moreover, established poor prognostic factors like high serum bð2-microglobulin levels, abnormal karyotype, high creatinine concentration and chromosome 13 deletions were more prevalent in the MM4 subgroup compared to the MM1 subgroup, suggesting that the MM4 subgroup represents a high-risk clinical entity. In the same study, a comparison between normal bone marrow plasma cells and myeloma plasma cells yielded 120 differentially expressed genes. The largest group of downregulated genes in MM plasma cells coded for signaling pathway associated molecules and adhesion associated molecules. Strikingly, the gene coding for CD27 was the second most downregulated gene in MM plasma cells compared to normal plasma cells, corroborating our findings[101]. A high (spiked) expression of the fibroblast growth factor receptor 3 (*FGFR3*) and cyclin D1 (*CCND1*) genes coincided with the t(4;14) and t(11;14) indicating that these translocations result in an increased expression of the involved (onco)genes located on the partner chromosomes. In another study, expression profiling was performed on purified plasma cells from 6 MM, 3 PCL patients, 8 MM cell lines and nonmalignant plasma cells derived from *in vitro* cultures of end-differentiated peripheral blood B cells.[138,139] A malignant plasma cell group and a normal plasma cell group was identified by hierarchical clustering of these samples. In this analysis 250 genes were significantly upregulated and 159 genes were significantly downregulated in malignant plasma cells. The upregulated genes coded for cell cycle associated proteins (e.g. Cyclin D1, CDC34), myc and myc-interacting proteins (e.g. BMI-1, GCN5L2), anti-apoptotic proteins (e.g. Bcl-2, IER3,HSP27), tyrosine kinases (e.g. ABL tyrosine kinase, Tyro3), metabolism proteins (e.g. CBS, PFKM), metastasis-associated proteins (e.g. SPARC) and established tumor antigens (e.g. MAGEs, NY-ESO, SSX2). Interestingly, the ABL tyrosine kinase, involved in the t(9;22) in chronic myeloid leukemia, was significantly upregulated in MM samples. De Vos *et al*. showed that simultaneous blocking of the ABL kinase

activity by STI571 and addition of anti-IL-6 antibodies significantly decreased the proliferation of an IL-6-dependent MM cell line. This nicely demonstrates that cDNA microarray analysis may identify (unknown) pathways that are dysregulated in MM, and might provide a rationale to target these pathways in therapeutical settings. Furthermore, this technique can be used to identify patients in which these pathways are deregulated and are therefore eligible for these (novel) treatment modalities.

In a recent study Zhan *et al.* compared expression profiles of MM samples with expression profiles obtained from B cells at distinct (late) developmental stages.[140] By hierarchical clustering a group of genes was found that distinguished tonsillar B cells from tonsillar plasma cells, termed early differentiation genes. Similarly, a group of genes was found distinguishing tonsillar plasma cells from bone marrow plasma cells, termed late differentiation genes. The earlier identified MM subgroups (MM2, MM3, MM4)[103] showed a striking variability in the expression of early and late differentiation genes. Importantly, regarding expression profiles, it was demonstrated that MM subgroups had distinct B cell developmental features. The MM4 subgroup (closely resembling MM cell lines) were most similar to tonsillar B cells, the MM3 subgroup to tonsillar plasma cells and the MM2 to bone marrow plasma cells. The MM1 subgroup did not significantly resemble any of the normal B cell developmental stages which were included in this study. These results again underscore the fact that heterogeneity in MM is intimately linked to the maturational stage of the plasma cells, recapitulating the morphological, phenotypical and molecular features of MM plasma cells.

Follow-up patient-studies are necessary to determine the predictive power and the clinical validity of cDNA microarray analysis and hierarchical clustering for MM.

CONCLUDING REMARKS

Although the tumor clone in MM patients is considered to comprise of primarily end-differentiated cells, we hypothesize that the tumor clone has not (yet) lost it differentiational capacity and thus might also include earlier differentiational B cell stages. Several studies show that a (relative) plasma cell immaturity imposes a poor prognosis and a worsened clinical outcome. Whether this is due to differences in the cell of origin or whether the tumor clone in MM displays maturational plasticity is uncertain. In other words, this plasticity could be due to intrinsic features of malignant plasma cells or may be attributable to the existence of a tumor-precursor population. In general, the studies concerning the heterogeneity in MM uniformly stress the importance of the plasma cell maturation stage and its implications on prognosis and clinical outcome. In summary, the heterogeneity of the MM tumor clone reflects the heterogeneity in the pathogenetic processes leading to the manifestations of clinical features which are characteristic for MM, but eventually might very well describe a composite group of patients, which should be differentially diagnosed and treated concomittantly.

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chapter three

Autologous stem cell transplantation in multiple myeloma after VAD and EDAP courses: a high incidence of oligoclonal serum Igs post transplantation

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SUMMARY

Thirty-seven patients with multiple myeloma (stage II and III, 65% increased β2-microglobulin level) were prospectively treated with a median of 3.7 VAD courses (range 2-8) followed by cyclophosphamide (6 g/m^2) in conjunction with G-CSF (5 μg/kg filgrastim (n = 14), or 3.5 μg/kg lenograstrim (n = 22)), and peripheral stem cell (PSC) isolation. After regeneration this was followed by one EDAP course and high-dose melphalan (HDM 200 mg/m^2) in combination with re-infusion of PSC. Adequate stem cell mobilization was obtained with both G-CSF regimens. A median of 41 x 10^6 CD34$^+$ cells/kg (range 4.5-161) was collected in a median of 1.6 leukapheresis procedures following filgragrastim (n = 14) and 24 x 10^6 CD34$^+$ cells/kg (range 2.3-80) in a median of 1.7 leukapheresis procedures following lenograstim (n = 22) which indicated no significant difference (P = 0.24) between both G-CSF regimens. A rapid hematological recovery was obtained after HDM with reinfusion of a median of 9.3 x 10^6 CD34$^+$ cells/kg. After the total courses the overall response was 84% with a complete remission rate of 30%. Currently the median overall survival is 44.0 months (95% CI 38.9-49.1) with a median follow-up of 33 months (range 3-51) and a median event-free survival of 29.0 months (95% CI 25.3-32.7). Post-transplantation a high incidence of oligoclonal serum immunoglobulins (Igs) was observed. In 73% of the patients new oligoclonal or monoclonal serum bands were noticed 3 months post transplantation. IgGλ and IgGκ bands predominated. In 48% of the cases the oligoclonal Igs disappeared after a median follow-up of 22 months (range 8-36), whereas in 52% of the cases the oligoclonal Igs persisted with a median follow-up of 31 months (range 21-45), which did not correlate with a significant difference in overall, and event-free survival between both subgroups.

INTRODUCTION

The treatment of multiple myeloma during the last three decades has consisted predominantly of alkylating agents. Long-term survivors are unusual with this treatment modality and the median survival duration is 24-36 months.[1,2] Further increase in dose intensity can obtained by performing autologous stem cell transplantation which can be facilitated by the use of peripheral blood stem cells isolated following cyclophosphamide treatment or granulocyte colony-stimulating factor (G-CSF) or granulocyte-macrophage-CSF (GM-CSF).[3-5] Limited data are available regarding the optimal dose of hematopoietic growth factor (HGF) support. Demuynck *et al*[6] reported recently that G-CSF at a dose of 10 μg/kg may be preferred because of its markedly lower toxicity and lower in-hospital costs in comparison to GM-CSF. However, it is not excluded that a lower dose of G-CSF might give similar results which can further reduce the costs related to the leukapheresis procedure. It was recently also suggested that oligoclonal serum Igs occuring post transplantation might be an independent prognostic factor for overall survival[7]. The oligoclonal serum Igs were shown in 10% of the patients following high-dose melphalan and/or total body irradiation. The present study was designed to define further the optimal dose of growth factor. In addition, the occurence of oligoclonal serum Igs were evaluated in the total group of patients. Post transplantation, new oligoclonal serum Igs were demonstrated in 73% of the patients.

PATIENTS, MATERIALS AND METHODS

Patients and response criteria

Between August 1993 and April 1996 all patients at our department with high risk multiple myeloma were prospectively treated with autologous stem cell transplantation. Thirty-seven patients were included according to the following criteria: (1) age less than 65 years; (2) stage II or III according to the staging system of Durie and Salmon[8] and (3) serum creatinine <160 μmol at the time of transplantation. Four patients were pretreated with two melphalan/prednisolone courses. Disease response was defined as follows: patients were regarded to have achieved complete remission (CR) when the original serum M-protein had disappeared, tested at least twice by immuno-electrophoresis according to Pascali *et al*;[9] no urine M-component measurable (immuno-fixation in concentrated urine) and less than 5% plasma cells in the aspirate with a polyclonal immuno-staining defined with anti-kappa and anti-lambda. Patients were in partial remission (PR) if there was a 50% decrease in measurable M-protein or bone marrow infiltration (non-secretory or Bence-Jones myeloma) compared with pretreatment values. Patients with less than 50% reduction in serum M-protein were considered as non-responders.

Oligoclonal serum Igs different from the monoclonal serum protein at presentation were detected as described above. The oligoclonal Igs were determined every 3-4 months post transplantation and had to be present on different occasions.

All patients received as the induction regimen vincristine (0.4 mg/m²) and doxorubicin (9 mg/m²) intravenously (i.v.) by continuous infusion for 4 days; dexamethasone (40 mg) orally days 1-4 and days 16-20 (VAD). This regimen was administered every 4 weeks until at least partial response (PR) was obtained or until the paraprotein level plateaued over two successive courses. In the case of responsive disease, patients were treated with high-dose cyclophosphamide i.v. (6 mg/m²) followed by granulocyte colony-stimulating factor (G-CSF). Leukapheresis was performed with a COBE-Spectra machine (COBE, Dallas, TX, USA) and started at a leukocyte count >2.0 x 10^9/l during the regeneration phase. The number of peripheral blood stem cells was identified by CD34-antigen expression measured by FACS analysis in combination with the number of colony-forming unit granulocyte-macrophage (CFU-GM) measured in an *in vitro* colony assay.[10] Two sources of G-CSF were used: lenograstim (Granocyte; Rhône Poulenc Rorer, Anthony, France) and filgrastim (Neupogen; Amgen, Thousand Oakes, CA, USA). Lenograstim is a human-identical glycosylated recombinant G-CSF whereas filgrastim is non-glycosylated recombinant-methionyl G-CSF. Lenograstim was given at a dose of 3.5 μg/kg/day and filgrastim was given at a dose of 5.0 μg/kg/day, both subcutaneously. Patients were prospectively randomized (lenograstim *vs* filgrastim). After normalization of peripheral blood counts patients were treated by etoposide 400 mg/m² i.v., cisplatinum 80 mg/m² i.v. on day 1, dexamethasone 40 mg orally for 4 days and ara-C 1000 mg/m² i.v. on day 5 (EDAP).[11] Finally, the treatment was followed by high-dose melphalan (HDM, 200 mg/m²) i.v. in combination with peripheral blood stem cell infusion. During the transplantation period patients were nursed in laminar air flow rooms according to the available facilities. Red blood cell transfusion was given at a hemoglobulin level of 8 g/dl or lower while a platelet transfusion was given at a platelet count of 10 x 10^9 or lower. No growth factor was applied after the infusion of peripheral blood stem cells.

Statistical analysis

The results of the study are based on an 'intention to treat' analysis. The Mann Whitney *U* test was used to define the difference between the growth factor combinations, the Kaplan-Meier product limit method for estimation of survival, and the log-rank test for comparison complete remission duration, event-free survival (EFS) and overall survival (OS).

RESULTS

Patient characteristics

Thirty-seven patients were included in this study. The patients' characteristics are listed in Table I. Median age was 51 years (range 36-65) with 23 men and 14 women. According to the Durie-Salmon staging system 5% of the patients had stage II-A, 78% stage III-A and 16% stage III-B. Increased β2-microglobulin (β2-M) levels were observed in 65% of the patients. The median β2-M level measured at presentation was 4.8 mg/l with a range of 1.0-17.6.

No. of patients	37
Age (years)	
Median	51.0
Range	36-65
Sex	
Female	14
Male	23
Stage	
IIA	2
IIIA	29
IIIB	6
Myeloma protein*	
IgG	21
IgA	5
IgM	1
BJ	7
Lambda	16
Kappa	18
PCL	3
Pretreatment	
No.	4
β2m (*n* = 31)	
Median	4.8
Range	1.0-17.6

Table I. Patients' characteristics at presentation.
PCL = plasma cell leukemia; β2m = β2-microglobulin, normal value 1.0-3.0 mg/l; BJ = Bence Jones. *The myeloma protein composition is described as % of the total.

Treatment

The patients were treated with a median of 3.7 (range 2-8) VAD courses. After the VAD courses, the overall response was 81% and 16% of the patients had a CR. Peripheral stem cell (PSC) collection was performed in all patients following high-dose cyclophosphamide (6 g/m^2) and G-CSF. Fourteen patients received filgrastim at a dose of 5.0 μg/kg whereas 22 patients received lenograstim at a dose of 3.5 μg/kg. The total number of days of growth factor administration varied with both regimens between 8 and 12 days. Adequate stem cell mobilization was obtained in all patients. A median of 41 x 10^6 CD34$^+$ cells/kg (range 4.5-161) was collected with the use of filgrastim in a median of 1.6 leukapheresis procedures (range 1-4). Comparable results were obtained with lenograstim. A median of 24 x 10^6 CD34$^+$ cells/kg (range 2.3-80) was collected in a median of 1.7 leukapheresis procedures (range 1-4). No significant difference was observed in the number of CD34$^+$ cells/leukapheresis (P = 0.24) between both regimens. A good correlation existed between the number of CD34$^+$ cells in the leukapheresis product and the number of CFU-GM (P = 0.016). Following the peripheral stem cell collection 36 patients were treated with one EDAP course. Response evaluation after EDAP and before transplantation demonstrated that 24% of the patients achieved a complete remission.

Autologous peripheral stem cell transplantation

Thirty-five patients received high-dose melphalan (200 mg/m^2) with support of autologous peripheral stem cells. Not all collected CD34$^+$ cells were used. This policy was followed to limit the possible load of infused malignant cells. A median of 9.3 x 10^6 CD34$^+$ cells/kg (range 2.3-30.8) was reinfused. A leukocyte count >0.5 x 10^9/l was reached after 13.9 days (range 10-20 days). The median duration of granulocytopenia (<0.5 x 10^9/l) and thrombocytopenia (<10 x 10^9/l) was 15.1 days (rnage 11-22 days) and 7.0 days (range 0-16 days), respectively. The

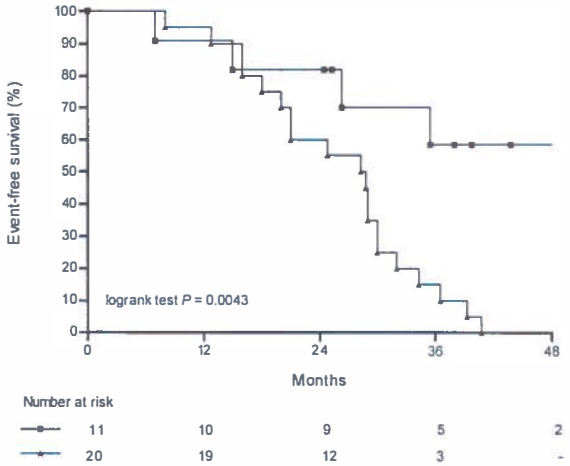

Figure 1. Kaplan-Meier estimates of distributions of event-free survival (EFS), based on 31 patients with multiple myeloma stage IIIA/IIIB (according to the staging criteria of Durie and Salmon). Median follow-up 33 months. CR n = 11; PR n = 20. A significant advantage in EFS was observed for patients with CR compared to PR (P = 0.004).

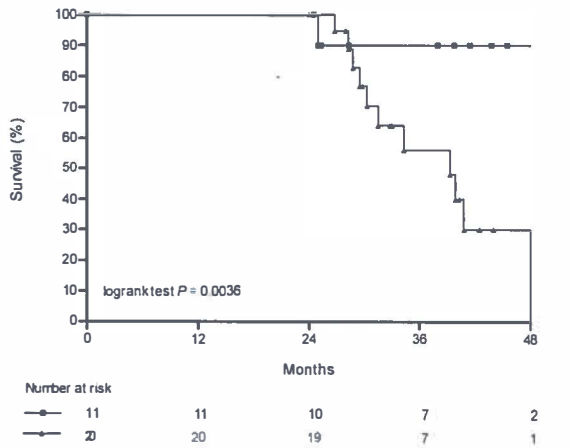

Figure 2. Kaplan-Meier estimates of distributions of overall survival (OS), based on 31 patients with multiple myeloma stage IIIA/IIIB (according to the staging criteria of Durie and Salmon). Median follow-up 33 months. CR n = 11; PR n = 20.

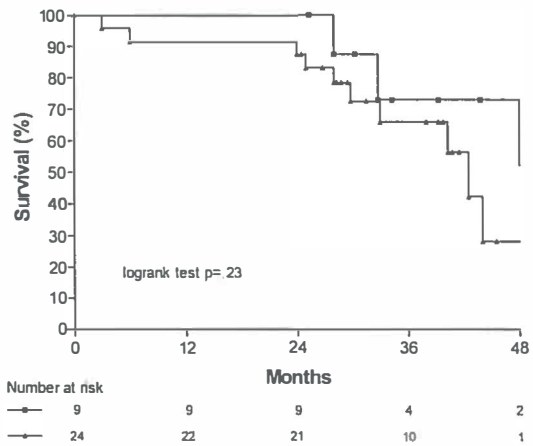

Figure 3. Kaplan-Meier estimate of distribution of overall survival (OS), based on 33 patients with (n = 24) and without (n = 9) oligoclonal gammopathies.

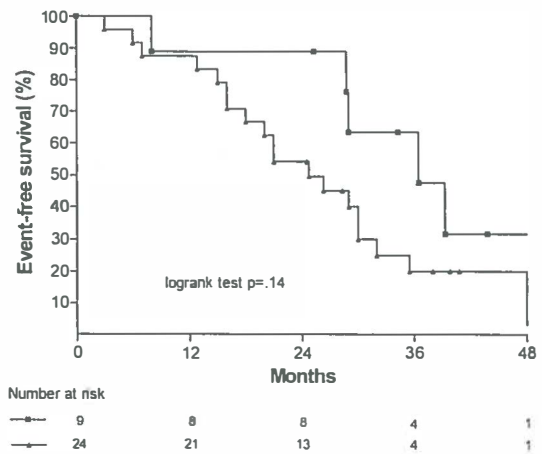

Figure 4. Kaplan-Meier estimate of distribution of event-free (EFS) based on 33 patients with (n = 24) and without (n = 9) oligoclonal gammopathies.

median duration of platelet count less than 20 x 10^9/l was 17.5 days (range 7-71 days). Three patients experienced a very prolonged period of thrombocytopenia of 40, 40 and 71 days, repectively. A median of 6.3 red blood cell transfusions was given (range 3-20) and a median of 2.6 platelet transfusions (range 0-8). The number of days with fever (defined as a temperature higher than 38°C) ranged from 0 to 16 days (median 5.2 days). During the regimen three patients died. One patient died during the pancytopenic phase after cyclophosphamide treatment as the result of sepsis. The other patients died during the transplantation period due to pneumonia and sepsis.

Long-term follow-up

Long-term follow-up could be evaluated in 33 patients. After transplantation 30% of the patients achieved a complete remission (CR) and 54% of the patients a PR. During a median follow-up of 33 months (range 3-51) 88.4% of the patients relapsed after a median of 28.6 months. A significant advantage in EFS was observed for patients with CR compared to PR (P = 0.004, Figure 1). The overall survival is 45% with a median survival time of 44.0 months (38.9-49.1, CI: 95%). Also a significant advantage in overall survival was observed for the CR patients compared to PR patients (P = 0.036, Figure 2).

Oligoclonality

In 33 patients it was possible to study the development of oligoclonal serum Igs in the post-transplant period after PSCT (Table II). New oligoclonal Igs appeared in 24 (73%) patients after a median of 3 months (range 1-6 months). Sixteen patients showed multiple bands and a single serum band could be identified in eight patients. Predominantly IgG-κ and IgG-λ gammopathies were noted each occurring in 38%. Nine cases showed IgM (18%), two cases IgA together with IgG and IgM. Lambda and kappa light chains appeared in 40% and 42% of the cases, respectively. Two patients with oligoclonal serum Igs in the post-transplant period died after 3 and 4 months, respectively, with the reappearance of the original malignant clone. In nine cases the oligoclonal serum Igs dissapeared spontaneously after a median of 22 months (range 8-36) post-transplantation. In all these patients the immunofixation became negative. The additional 15 patients still had oligoclonal serum Igs with a median follow-up of 31 months (range 21-45). In the group of patients with oligoclonal serum Igs 10 patients (42%) died with a median survival of 35 months (range 24-49). The mean survival of patients without oligoclonal serum Igs was not significantly different (P = 0.23) with this limited number of patients (Figures 3 and 4).

Table II. Type of oligoclonal serum immunoglobulins in 24 patients post transplantation.

	Total	*Transient*	*Persistent*
IgG-κ	19	8	11
IgG-λ	19	9	10
IgM-κ	2	1	1
IgM-λ	1	1	0
IgM	6	4	2
IgA	2	1	1
Bence Jones λ	1	0	1

DISCUSSION

This study represents patients with high-risk multiple myeloma treated with high-dose melphalan supported by PBSC after induction therapy with VAD and EDAP courses. A total of 37 patients have been studied, most of them with advanced stage disease at presentation and three of them with plasma cell leukemia. A CR rate of 30% was obtained after the total treatment period. The decline in tumor activity was most pronounced after the VAD courses. The peripheral blood stem cells could easily be collected after high-dose cyclophosphamide and G-CSF after one to two leukapheresis procedures. No advantage was observed for 5 μg/kg filgrastim over 3.5 μg/kg lenograstim. With both forms of G-CSF high numbers of $CD34^+$ cells were harvested in most cases, indicating that a much lower dose of G-CSF can be applied, as recently advised.[6] This will reduce further the costs related to the leukapheresis procedure. These data also reveal that the dose of growth factor used for stem cell mobilization seems to depend on the underlying disorder and chemotherapy. Recently, it was shown in acute myeloid leukemia that a dose of 400-600 mg/m^2 lenograstim is required for collecting an adequate stem cell transplant after the combination chemotherapy of amsacrine and cytarabine.[15]

A high incidence of oligoclonal serum Igs was observed post-transplantation. In 73% of the patients oligoclonal serum Igs appeared after a median of 3 months post transplantation. In most cases IgG bands were observed. In the minority IgM or IgA bands were observed. The presence of a oligoclonal B-cell proliferation is frequently noticed after allogeneic bone marrow transplantation.[16] In autologous bone marrow transplantation it has been noticed but the reported incidence is much lower as observed in the present study.[17-20] The difference in frequency of oligoclonality between the various studies is unclear. The high incidence in the present study seems not to be related to a difference in the CR rate as was recently suggested by Zent *et al.*[7] The appearance of oligoclonal serum Igs might be related to the strategy used providing a selective advantage to a population of B cells producing alternative isotypes. Alternatively, the sensitivity of the assay might be responsible for the difference in frequency

of oligoclonal serum Igs. Recently, we have shown by ASO-PCR and sequencing that oligoclonal serum Igs post transplantation is not caused by myeloma related clonal B cell but rather by the regenerating B cell compartment, indicating that the oligoclonal serum Igs post transplantation can not be considered as a sign of relapse of the disease.[21]

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chapter four

Multiple myeloma related cells in patients undergoing autologous peripheral blood stem cell transplantation

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SUMMARY

A high incidence of oligoclonal serum M-components is observed in Multiple Myeloma (MM) patients treated with autologous stem cell transplantation (ASCT). To determine whether these M-components are produced by myeloma clonally related cells or caused by an aberrant B-cell regeneration we analysed by semi-nested ASO-RT-PCR and DNA sequencing the immunoglobulin (Ig) variable genes (VH) obtained from bone marrow samples obtained before and after transplantation and peripheral blood stem cell (PBSC) samples from seven patients.

Myeloma clonally related cells are identifiable by the expression of variant Ig heavy chain isotypes and were detected in two patients at presentation. No myeloma clonally related cells were found in post-transplantation samples (n = 7) in spite of the appearance of new serum M-components. However, in two cases we amplified sequences from post-transplantation bone marrow cells that were able to bind to the B-cell clone-specific CDR3 oligonucleotides but showed no further similarity regarding the VDJ rearrangement. These data indicate that serum oligoclonality post-transplantation is not caused by myeloma clonally related B-cells but rather by the regenerating B-cell compartment.

INTRODUCTION

Multiple myeloma (MM) is a lymphoproliferative disorder characterized by the clonal expansion of plasma cells in the bone marrow, and the secretion of high amounts of monoclonal immunoglobulins (serum M-component). Characteristically, the neoplastic bone marrow plasma cells in multiple myeloma have undergone switch recombination and most patients secrete IgG or IgA.

The median survival of MM patients treated with conventional chemotherapy is 24-36 months.[1] Autologous stem cell transplantation (ASCT) is now a widely applied therapy for MM, which might prolong long-term survival.[2,3] Post-transplantation, the monoclonal serum protein disappears in 35% of the patients undergoing ASCT.[4] However, a high incidence of serum oligoclonality has been noted. In addition to variant heavy chain isotypes, alternative light chain isotypes are frequently observed.[5] The cause of the appearance of variant heavy and light chain isotypes is unknown, but might be caused by aberrant B-cell regeneration as seen in patients following allogeneic bone marrow transplantation.[6] Alternatively, the isotype variants in MM patients might be the result of the selection of myeloma clonally related cells. In order to investigate the origin of the newly observed monoclonal serum proteins we determined residual myeloma cells and myeloma clonally related cells expressing variant isotypes in peripheral blood stem cells (PBSC) and post-transplantation bone marrow cells from seven patients by using ASO-RT-PCR and DNA sequencing.

Myeloma clonally related cells expressing variant immunoglobulin heavy chain isotypes were identified only in pre-transplantation bone marrow and peripheral blood samples. These cells were never detected post-transplantation, therefore it is unlikely that new serum M-components are produced by myeloma clonally related cells post transplantation.

MATERIALS AND METHODS

Multiple myeloma patient samples

Patients were diagnosed multiple myeloma and staged according to the Durie and Salmon criteria.[7] Bone marrow aspirates at presentation, after ASCT and peripheral blood stem cell collections (PBSC) were obtained from seven patients. Mononuclear cells were isolated by Ficoll-hypaque density centrifugation (Lymphoprep™ Nycomed, Oslo, Norway). The patient characteristics are shown in Table I.

Autologous stem cell transplantation

Patients were treated with three or four courses of vincristine, adriamycin and dexamethasone (VAD) followed by high-dose cyclophosphamide (6 g/m^2), in combination with G-CSF (5 μg/kg Neupogen™ or 3.5 μg/kg Granocyte™ and peripheral stem cell isolation. After regeneration this was followed by a single course of etoposide, dexamethasone, Ara-C and cisplatin (EDAP), followed by high-dose melphalan (HDM, 200 mg/m^2) and PBSC reinfusion.

Table I. Patient characteristics. Abbreviations: CR, complete remission; PR, partial remission; n.d., not determined. * Patients were staged according to the Durie and Salmon criteria. † Post-transplantation serum M-components were determined 3-6 months after transplantation.

Patient	Stage	Relapse	Pre-transplantation M-component isotype/level	Post-transplantation M-component isotype/level†
1	3a	Patient died	IgGλ: 91.1 g/l	IgMκ: 2.0 g/l IgGκ: 35.5 g/l IgAκ: 3.2 g/l
2	3a	Stable	IgAλ: 42.8 g/l	IgMκ: 7.2 g/l IgGκ: 12.4 g/l
3	3a	CR	IgGκ: 23.9 g/l	IgGκ: 14.9 g/l IgGλ: n.d.
4	3a	Relapse	IgGκ: 60.7 g/l	IgGλ: 9.8 g/l
5	3a	Relapse	IgGκ: 46.0 g/l	IgGλ: 53.5 g/l
6	3a	Stable (PR)	IgGκ: 69.0 g/l	IgGκ: 19.8 g/l
7	3a	Stable (PR)	IgGκ: 70.0 g/l	Not detectable

Detection of serum M-components

Serum electrophoresis was performed and M-components were identified as described.[8]

RNA isolation and first-strand cDNA synthesis

Total RNA was isolated from approximately 10 x 10^6 frozen myeloma bone marrow mononuclear cells (MNC) and PBSC using Trizol™ according to the manufacturer's directions (Gibco BRL, Gaitherburg, Md.). RNA was resuspended in 100 µl DEPC treated MilliQ. Aliquots of 20 µl RNA (approximately 5 µg) were primed with 160 ng random hexamer primer (Pharmacia Biotech, Upsala, Sweden) and incubated with 0.5 mmol/l of each deoxynucleotide triphosphate at 70°C for 10 min. The primed RNA was reverse transcribed in a 30 µl reaction containing 200 U Moloney murine leukaemia virus RT(SuperscriptII™ Gibco BRL), 50 mmol/l KCl, 20 mmol/l Tris-HCl, pH 8.4, 2.5 mmol/L $MgCl_2$ and 1 mmol/L DTT (Gibco BRL) for 30 min at 45°C. The reaction was stopped by enzyme inactivation at 95°C for 5 min. The reaction mixture was phenol extracted and ethanol precipitated. The cDNA was resuspended in 20 µl MilliQ.

Amplification and sequencing of myeloma VH genes

Biotinylated FR1 oligonucleotides originally designed for the VH3 (5'-XGAGGTGCAGCTGGTGGAGTCTGG-3') and VH4 (5'-XCAGGTGCAGCTGCAGGAGTCGGG-3') gene families were used together with Cγ (5'-GGGTCTAGACAGGCAGCCCAGGGCCGCTGTGC-3') or Cα (5'-GCTCAGCGGGAAGACCTTGG-3') IgH constant region oligonucleotides to amplify expressed VH genes in the bone marrow pre-transplantation. PCR reactions were performed in 50 μl containing 20 mmol/l Tris-HCl, pH 8.4, 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 30 pmol of each primer, 0.1 mmol/l of each deoxynucleotide triphosphate, 2.5 U Taq polymerase (Gibco BRL), 0.05% (w/v) W-1 and 4 μl of the first-strand cDNA reaction material. The first PCR cycle consisted of 2 min denaturation at 94°C, 2 min annealing at 60°C and 2 min extension at 72°C. This was followed by 34 cycles of 1 min steps at the above temperatures, with a final 10 min extension phase at 72°C. Amplified DNA was separated on 1.2% agarose gel and visualized by UV light after ethidium bromide staining. Amplified DNA was subjected to direct single-strand sequencing using avidin-coated beads (Dynabeads™ Dynal, Oslo, Norway) and the dideoxy chain termination method as described.[9] Sequencing gels were analysed on an automated laser sequencing device (Pharmacia biotech). FITC-labelled Ig constant region specific sequencing oligonucleotides were used (cγ: 5'-XGTT/CGCCAGGGGGAAGAC-3'; Cα: 5'-XGCTCAGCGGGAAGACCTTGG-3'). Direct sequencing of pre-transplantation bone marrow PR-products yielded only interpretable sequences when the PCR-product was dominated by the malignant clone because of the monoclonal nature of pre-treatment myeloma bone marrow. VH genes were identified by comparing the sequences with the V-base sequence database.[10]

ASO-RT-PCR

We designed allele-specific oligonucleotides (ASO) based on the VH complementarity determining regions (CDR) 1 and 3 to identify residual myeloma cells and myeloma clonally related cells. In a semi-nested PCR strategy we paired IgH constant region specific oligonucleotides (Cμ: 5'-GAGGATCCGGGTGCTGCTGATGTCAGA-3'; Cγ, Cα: see above) with CDR1 oligonucleotides in the first-round PCR, and subjected 1 μl of PCR product to second-round PCR with the IgH constant region oligonucleotides and CDR3 oligonucleotides. CDR1 oligonucleotides were chosen to end on a somatic mutation whereas CDR3 oligonucleotides were chosen in the VH-D junctional area to assure high specificity. Primer melting temperatures were between 57°C and 68°C. Primer specificity was confirmed by performing PCR on healthy donor bone marrow MNC and other myeloma patient bone marrow MNC. Oligonucleotides were accepted only if reactivity with the correct patient was observed. PCR products of interest were cloned into the pCRII vector using the TA cloning system (Invitrogen, Carlsbad, Calif.). Plasmids containing inserts of the correct size were sequenced by cycle sequencing using plasmid-specific oligonucleotides (M13 forward: 5'-GTTTTCCCAGTCACGAC-3'; M13 reverse: 5'-CAGGAAACAGCTATGAC-3').

Table II. Allele-specific oligonucleotides.

Patient	CDR1 ASO
1	5'-GCAAGGCGTCTGGTTACA**CGTTCACCGGCTACTATGTC**CACTGGG-3'
2	5'-TGTGCAG**CCTCGAATTCACCATTAGAG**GCTATGCCATGAGCTGGA-3'
3	5'-GTGCCATC**TCCGGGGACAGTGTCTATAA**CAACAGTGCTGCTTGGA-3'
4	5'-TTGCAGC**CTCGGGATTAACCTTCAATG**ACTATAGCATGCACTGGG-3'
5	5'-GGCAACCY**TCTGGATTCACCCTTTCACTAG**TACTGGATGCACTGGG-3'
6	5'-GTGCAGCCTCT**GGATTCACGTTTGATGATCAC**GCCATTCACTGGG-3'
7	5'-GTACAGGCTC**TGGATTCCCCTTCAGTGCT**TATGCTCTGCACTGGG-3'

Framework 1-complementarity determining region 1-framework 2 sequences obtained from diagnosis bone marrow samples are listed above. Complementarity determining region 1 allele-specific oligonucleotides designed for each patient are represented as bold and underlined sequences.

Patient	CDR3 ASO
1	5'-ACGGCCGTCTA**TTATTGTGCGAGAGG**AGGCGGGGGGGCATCA-3'
2	5'-ACGGCCCTA**TATTACTGTGCGGATGGG**GGCCCTAATAGTGGC-3'
3	5'-ACGGC**TGTATATTACTGTGTAAGAGACG**CCACCAAACTCGTC-3'
4	5'-ACGGCTGTTTATTACTGTGTG**AGAGATCAAGAGCGGAGATTT**TCG-3'
5	5'-ACGGCTGTATATTACT**GTGCAAGAGGAAGCTCATAC**TCCACC-3'
6	5'-ACGGCCCTGTATCATTGTGCAAAAGATATCG**GGTCCCTCGGAATAGAT**CCA-3'
7	5'-ACGGCTGTATATTACTGTGTGAGAGCCCCGGA**ATCTCCGAATCTAGCAACA**GCTGC-3'

IgH variable region gene segment-diversity gene segment junctional sequences obtained from diagnosis bone marrow samples are listed above. Complementarity determining region 3 allele-specific oligonucleotides designed for each patient are represented as bold and underlined sequences.

FACS sorting

To identify myeloma clonally related cells expressing variant light chain isotypes, we sorted post-transplantation bone marrow mononuclear cells on the basis of their κ and λ expression and subjected both cell populations to semi-nested ASO-RT-PCR. Cells were stained with anti-human κ-FITC or anti-human λ-FITC MoAb (DAKOpatts, Glostrup, Denmark) and were analysed on a FACStar® instrument (Becton Dickinson, Mountain View, Calif.). Purity of sorted cells was confirmed by κ/λ specific RT-PCR.

RESULTS

The bone marrow samples obtained prior to treatment were shown to contain a dominant clonal VH rearrangement, expressed as an IgG (patients 1, 3, 4, 5, 6 and 7) or an IgA (patient 2) isotype. Sequences were submitted to the EMBL databank under accession numbers AJ007438-AJ007444. CDR1 and CDR3 allele-specific oligonucleotides designed on the basis of these sequences are shown in Table II.

We examined pre-treatment bone marrow cells and peripheral blood samples from seven patients for the presence of clonally related cells expressing variant Ig isotypes. In samples from two patients (nos. 3 and 6) we detected cells that expressed different Ig isotypes pre-transplantation (Table III).

Patient 3 had an IgG-κ serum M-component pre-transplantation whereas, with ASO-RT-PCR, IgM and IgA expressing cells were detected that share the Ig heavy chain variable region expressed by the malignant IgG myeloma cells. Sequences of the variable regions linked to the different Ig heavy chain constant regions expressed by these cells are depicted in Fig 1(a).

Patient 6 had an IgG-κ serum M-component at presentation whereas ASO-RT-PCR demonstrated the presence of IgA-expressing cells which share the Ig heavy chain variable region expressed by the malignant IgG myeloma cells (Fig 1b).

Contamination with residual myeloma cells were shown in PBSC samples from 4/7 patients (Table III). Myeloma cells expressing Ig isotypes distinct from the pre-transplantation serum M-component were not detected.

In post-transplantation bone marrow samples from 6/7 patients we found myeloma cells (Table III). Myeloma cells with altered Ig heavy chain isotype expression were not detected in post-transplantation bone marrow using ASO-RT-PCR (n = 7) (Table III).

Table III. Results ASO-RT-PCR. Clonally-related isotype variants are characterized by the expression of different Ig isotypes sharing the IgH variable region expressed by the malignant myeloma cells and were detected by Ig isotype-specific ASO-RT-PCR. * Clonally-related isotype variants. † CDR3-Cγ PCR-product was only detected using κ-sorted cell population. ‡ Myeloma non-related clones.

	Pre-transplantation			Peripheral blood stem cells			Post-transplantation			
Patient	IgM	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA	NCR‡
1	-	+	-	-	+	-	-	+	+	-
2	-	-	+	-	-	+	-	-	+	+
3	+*	+	+*	-	-	-	-	+†	-	-
4	-	-	+	-	-	-	-	+	-	-
5	-	+	-	-	+	-	-	+	-	-
6	-	+	+*	-	+	-	-	+	-	-
7	-	+	-	-	-	-	-	-	-	-

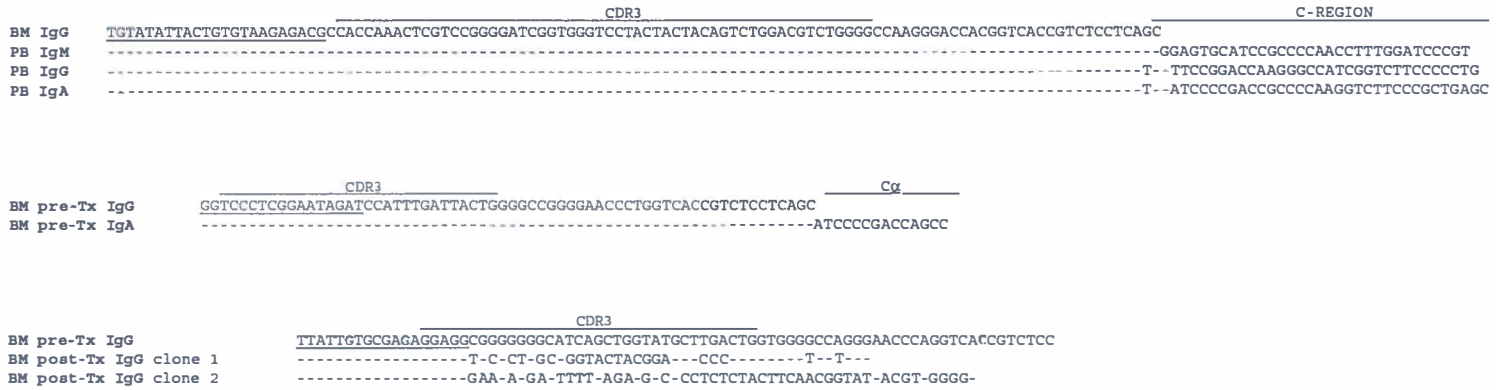

Figure 1. (a) Patient 3: sequences from CDR3-Cμ, Cγ, Cα PCR products obtained from pre-transplantation PB, compared to sequence obtained from pre-transplantation BM. CDR3 primer is underlined; CDR3 region and IgH constant region are indicated above sequence. (b) Patient 5: sequences from CDR3-Cγand CDR3-Cα PCR products obtained from pre-transplantation BM. CDR3 primer is underlined; CDR3 region and Cα region are indicated above sequence. (c) Patient 1: sequence from CDR3-Cγ PCR products obtained before and after treatment. CDR3 primer is underlined; CDR3 region is indicated above sequence. Identical bases are shown as dashes.

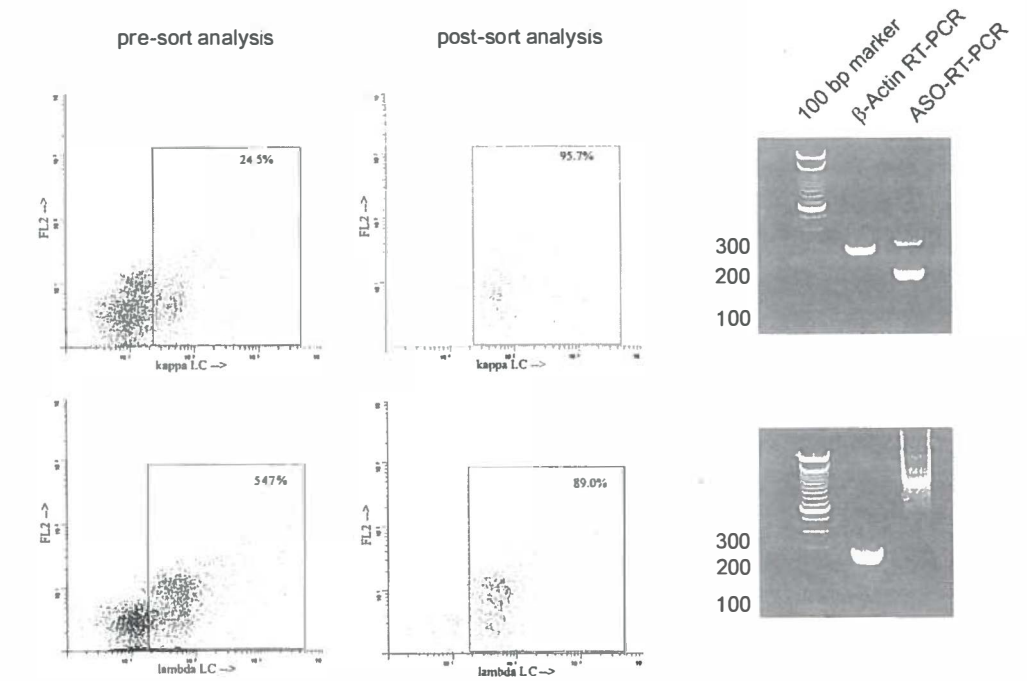

Figure 2. (a) Patient 3: flow cytometric analysis of sorted post-transplantation bone marrow cells. Cells were stained with anti-Kappa-FITC (upper panels) or anti-Lambda-FITC (lower panels) moab. FACS analysis of unsorted cells and re-analysis after sorting is shown. β-Actine control RT-PCR was performed on sorted populations, expected size of β-Actin PCR product is 290 basepairs. CDR3-Cγ PCR product was detected only in the Kappa-positive cell population. Expected size of CDR3-Cγ PCR product is 187 basepairs.

In 5/7 patients (nos. 1-5) serum Ig light chain expression was altered post-transplantation. Patient 3 had and IgG-κ serum M-component at presentation and IgG-κ and IgG-λ serum M-components post-transplantation. To determine whether the newly observed IgG-λ serum M-component was derived from myeloma clonally related cells, bone marrow cells were sorted in κ and λ positive cell populations and subjected to the semi-nested ASO-RT-PCR (Figure 2). Myeloma cells were only found in the κ positive cell population (Table III).

In post-transplantation bone marrow samples from 2/7 patients (nos. 1 and 2) we found B cells which expressed Ig sequences able to bind the CDR3 oligonucleotides in a non-nested ASO-RT-PCR but which were clearly not clonally related to the myeloma cells. Two non-myeloma IgG B-cell clones expressing Ig sequences able to bind the CDR3 oligonucleotide were found in the post-transplantation bone marrow sample from patient 1 (Fig 1c). A non-myeloma IgM B-cell clone expressing Ig sequences able to bind the CDR3 oligonucleotide was detected in the post-transplantation bone marrow from patient 2 (Table III).

DISCUSSION

The appearance of new serum M-components of different isotypes post-transplantation gives rise to the question whether myeloma clonally related cells expressing variant isotypes are present in the patient after ASCT. Alternatively, the new monoclonal Igs could be produced by normal B cells that regenerate aberrantly following transplantation. Several groups have demonstrated the presence of cells expressing malignant plasma cell VDJ rearrangements linked to the Cμ sequence in bone marrow and peripheral blood prior to treatment.[11,12] In addition, myeloma clonally related cells expressing different Ig isotypes can be detected in the bone marrow at presentation and can be distinguished an the basis of the expression of the membrane markers CD38 and CD45.[11,12]

We detected myeloma clonally related cells in 2/7 patients prior to treatment (patient 3 and 6). In the peripheral blood sample from patient 3 IgM, IgG and IgA expressing cells were detected in the bone marrow. In contrast, in no cases of myeloma clonally related cells expressing different Ig isotypes were found post-transplantation, despite the presence of new serum M-components. These data suggested that the new serum Igs post-transplantation were produced by non-malignant B cells in the regenerating bone marrow and cannot be considered a reliable tool to predict the early onset of relapse.

B cells that expressed sequences that could bind the CDR3 oligonucleotide but did not belong to the myeloma clone were detected by ASO-RT-PCR in post-transplantation bone marrow cells. These cells were only detected by using a non-nested ASO-RT-PCR. The occurrence of a B-cell clone expressing a CDR3 with substantial sequence similarity is statistically small. It is conceivable that a reduced B-cell repertoire which is observed post-transplantation[13] is responsible for the detection of such a B-cell clone. However, these cells were not detected with the more stringent semi-nested ASO-RT-PCR which requires the combination of CDR1 and CDR3 specificity. These data indicate that for an accurate detection of myeloma (clonally related) cells nested ASO-RT-PCR is required.

In summary, this study presents data showing that new serum M-components post-transplantation are not produced by myeloma related cells but rather by normal B cells, and reflect the recovery of B-cell function in these patients. This is also supported by the recent findings of Zent *et al*[4] who showed that MM patients with oligoclonal protein bands post-transplantation have a higher overall and event-free survival.

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chapter five

Myeloma clonotypic B cells are hampered in their ability to undergo B cell differentiation *in vitro*

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SUMMARY

In the peripheral blood (PB) of Multiple Myeloma (MM) patients clonotypic B cells are present that express identical V(D)J rearrangements as the malignant plasma cells in the bone marrow. In the present study, the proliferative capacity of clonotypic B cells from MM patients (n = 10) and the ability to differentiate *in vitro* was determined using the CD40-culturing system. For six patients, the presence of clonotypic B cells expressing variant immunoglobulin (Ig)-isotypes was assessed by Ig isotype-specific allele-specific oligonucleotide reverse transcription polymerase chain reaction (ASO-RT-PCR) after culturing with CD40L and interleukin 4 (IL-4). In three out of six patients, clonotypic B cells expressing variant isotypes were detected both before and after culturing. The ability of clonotypic B cells to undergo B cell differentiation was studied by abrogating CD40 signaling accompanied by IL-10 and IL-2 stimulation, enhancing differentiation towards Ig-secreting cells. The numbers of clonotypic B cells were determined by quantitative ASO-PCR. An increase in cell number was observed upon CD40L and IL-4 stimulation, whereas the relative number of clonotypic B cells was unaltered. In contrast, upon B-cell differentiation the relative number of clonotypic B cells decreased. In conclusion, clonotypic B cells can be cultured and isolated *in vitro* using the CD40 system. Clonotypic B cells responded to CD40 triggering in a similar fashion as non-clonotypic normal B cells. However, the ability of clonotypic B cells to undergo *in vitro* activation and differentiation into Ig-secreting cells is hampered.

INTRODUCTION

Multiple Myeloma (MM) is a neoplastic B cell disorder characterized by a clonal expansion of malignant plasma cells in the bone marrow. Although high-dose chemotherapy in combination with autologous stem cell transplantation has improved the event-free and overall survival, prognosis is still poor in MM.[1,2] The relatively limited growth capacity of myeloma plasma cells led to the assumption that more immature B cells might be involved in myelomagenesis. Circulating clonotypic B cells might represent the proliferative and disseminating myeloma-precursor cell population. Several groups have shown that clonotypic B cells are present in the bone marrow and peripheral blood of MM patients, using immunoglobulin (Ig) gene rearrangement analysis on sorted B-cell populations[3-6] and by single-cell (*in situ*) RT-PCR.[7,8] In contrast to the malignant plasma cells in the bone marrow, clonotypic B cells functionally express the multidrug transporter molecule MDR1 (Pgp170), suggesting that clonotypic B cells in MM reflect a chemoresistant precursor population responsible for relapse of the disease.[9,10] Furthermore, Pilarski *et al.* (2000) showed that NOD/SCID mice xenotransplanted with granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood stem cells from MM patients, exhibit MM-like characteristics including lytic bone lesions, human plasma cells in murine bone marrow and human Ig in the serum, indicating that circulating clonotypic cells have myelomagenic abilities.[11] However, there is no direct evidence from these experiments that clonotypic B cells and not plasma cells are responsible for the observed phenomena. These data indicate that the role of clonotypic B cells in the malignant process of MM is as yet poorly defined and that the origin and nature of clonotypic B cells remains to be elucidated. The unique Ig rearrangement expressed by the malignant plasma cells provides important information regarding the origin of MM. Importantly, the presence of somatic hypermutations in a non-random fashion in the variable regions of the Ig rearrangement suggests that myeloma cells have undergone antigenic selection.[12-15] Additionally, we and others have shown that clonotypic B cells expressing different Ig isotypes are present in MM patients by allele-specific oligonucleotide reverse transcription polymerase chain reaction (ASO-RT-PCR), suggesting a post-germinal centre pre-Ig isotype switch origin for MM (e.g. memory B cell or plasmablast).[16-18] Although the presence and the number of clonotypic B cells have been described extensively, the functional aspects of clonotypic B cells are still poorly defined. In this report we set out to functionally characterize clonotypic B cells by determining their presence and number under different culturing conditions *in vitro.* In this study, it was shown that myeloma clonotypic B cells proliferate in response to CD40 ligation in a similar fashion as normal B cells do. However, myeloma clonotypic B cells did not respond to triggers involved in B cell differentiation, suggesting that clonotypic B cells differ from normal B cells regarding their capacity to respond to these triggers.

MATERIALS & METHODS

Multiple Myeloma patients' samples

Patients were diagnosed and staged according to the Durie and Salmon criteria.[19] After informed consent, peripheral blood was collected and bone marrow cells were obtained from iliac crest. Mononuclear cells (MNC) were isolated by Ficoll-Paque density centrifugation (1.077 g/ml, Amersham-Pharmacia Biotech, Sunnyvale, CA) and cryopreserved in liquid nitrogen until used.

Cloning of patient-specific Ig VH genes and design of allele specific oligonucleotides

Total RNA was isolated from 2 x 10^6 bone marrow mononuclear cells using Trizol™ reagent (Life Technologies, Glasgow, Scotland) according to the manufacturers instructions. Approximately 1 μg of RNA was primed with 165 ng random hexamers (Amersham-Pharmacia Biotech) and reverse transcribed in 20 μl containing 200 U Moloney murine leukaemia virus reverse transcriptase (SuperscriptII™ Life Technologies), 50 mmol/l KCl, 20 mmol/l Tris-HCl (pH 8.4), 2.5 mmol/l $MgCl_2$, 1 mmol/l dithiothreitol (DTT) and 0.5 mmol/l of each deoxynucleotide triphosphate (dNTPs), for 50 min at 42°C. The reaction was stopped by enzyme inactivation for 5 min at 95°C.

Ig VH genes were amplified using VH family-specific FR1 and Ig-constant region specific oligonucleotides as described.[20] Briefly, 0.5 μl cDNA was subjected to a 25 μl PCR reaction containing 10 mmol/l Tris-HCl (pH 8.4), 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 200 mmol/l of each dNTP, 2.5 U Taq DNA polymerase (Life technologies) and 30 pmol of each primer (Life Technologies). PCR cycles were preceded by a 3 min incubation at 95°C. PCR cycling conditions were as follows: 30 cycles of 30 s at 94°C, 30 s at 60°C and 1 min at 72°C, and finally a 10 min extension phase at 72°C in a Thermolyne Amplitron II™ (Dubuque, IO, USA). PCR-products were separated on a 2% LE-agarose gel (Seakem, FMC Bioproducts) and stained with Gelstar™ (FMC Bioproducts).

PCR-products were cloned directly into the pCR2.1TOPO™ vector (Invitrogen, Leek, the Netherlands) according to the manufacturer's instructions. Positive clones were identified by blue/white screening and restriction enzyme analysis. Plasmids were isolated using the High-Pure plasmid isolation kit (Roche Molecular Biochemicals, Mannheim, Germany). A minimum of eight clones was partially sequenced using only the ddTTPs ('T-tracks') to identify clones in which the dominant tumour-derived IgH-sequence was present. These clones were then cycle sequenced with the complete set of ddNTPs using fluorescein isothiocyanate (FITC)-labelled plasmid-specific oligonucleotides (M13forward and M13reverse). Sequencing gels were analysed using the ALFwin automated sequencing device (Amersham-Pharmacia Biotech). DNA sequences were compared to the Vbase germline VH sequences [21] using DNAPLOT software. ASO for CDR1 and CDR2 were designed to preferentially bind a somatic mutation at the 3' side. CDR3 oligonucleotides were chosen to end in the N-region from the VH-D junction. Primer specificity was confirmed by performing PCR on bone marrow obtained from healthy donors and other MM

patients. Sequences were submitted to European Molecular Biology Laboratory (EMBL) database and are available under accession numbers Y08401 (patient no. 1), AJ007440 (patient no. 2), AJ309825 (patient no. 3) X95661, (patient no. 4), AJ309826 (patient no. 5), X95659 and X95660 (patient no. 6), AJ305228 to AJ305231 (patients nos. 7 to 10).

Culturing peripheral blood B cells in the CD40 system

Frozen peripheral blood (PB) samples were thawed in newborn calf serum (NCS, Life Technologies) and washed twice in Hank's balanced salt solution (HBSS; Life Technologies). B cells were enriched by rosetting with 2-aminoethylisothiouronium-hydrobromide (AET; Sigma Chemicals, St. Louis, MO) treated sheep red blood cells (SRBC). MNC (2 x 10^6/ml) were incubated for 1 h with an equal volume of 5% AET-SRBC at 4°C. Rosetted T cells were depleted by Ficoll-Paque density centrifugation. Cells were washed twice with HBSS prior to culturing. T cell-depleted PBMNC (4 x 10^4) were cultured in flat-bottomed 24-well plates (Nalge Nunc International, Roskilde, Denmark) in the presence of 4 x 10^4 irradiated (100 Gray) 3T6 mouse fibroblasts transfected with human CD40L (3T6CD40L). Culture medium consisted of supplemented Iscove's-modified Dulbecco's medium (IMDM; Life Technologies) with 5% fetal calf serum (FCS; Life Technologies), 2 g/l bovine serum albumin (BSA fraction V; Sigma Chemicals), 10^{-5} mol/l β-mercaptoethanol, $3x10^{-5}$ mol/l ethanolamine (Sigma Chemicals), 40 μg/ml transferrin (Roche Molecular Biochemicals) 5 μg/ml insulin and 10 ng/ml recombinant human (rh)IL-4 (Peprotech CE, London, U.K.). Medium was replaced twice weekly and irradiated 3T6CD40L were added weekly. After 7 or 14 d, separate wells were analysed by semi-nested IgH isotype-specific ASO-RT-PCR. In the first step PCR, patient-specific CDR1 or CDR2 and IgH isotype-specific oligonucleotides were used in a 25 μl PCR reaction. In the second step, 0.5 μl PCR-product was amplified with CDR3 and IgH isotype-specific oligonucleotides. PCR-products were separated on a 3% agarose-gel (Metaphor™ FMC bioproducts).

From an additional patient (number 8) PB cells were subcloned *in vitro* after 14 d of bulk culturing. For subcloning, 10 cells were seeded onto 10^3 irradiated 3T6CD40L and rhIL-4 (10 ng/ml) in flat-bottomed 96-well plates (Nalge Nunc International). Fresh medium was added twice weekly, irradiated 3T6CD40L cells were added weekly. Subclones were analysed by IgH isotype-specific ASO-RT-PCR after 28 d of culturing.

Southern Blotting

CDR1-Cμ, CDR1-Cγ and CDR1-Cα RT-PCR products amplified from clonotypic B cells which were subcloned in the CD40 culture system (patient no. 8), were blotted onto positively charged membranes (Hybond™ Genescreen, Boston, MA). Blots were hybridized with a CDR3 probe, which was tailed with digoxygenin using a dig-tailing kit (Roche Molecular Biochemicals). The membranes were hybridized in 5 x standard saline citrate (SSC) + 0,1% N-lauroylsarcosine + 0,02% sodium dodecyl sulphate (SDS) + 0,1% blocking reagent (Roche Molecular Biochemicals)

at 54°C. Blots were developed with horseradish peroxidase-conjugated rabbit-anti-digoxygenin F(ab') fragments and the chemoluminescent substrate CSPD™ (Roche Molecular Biochemicals).

In vitro B cell differentiation

PB B cells from untreated MM patients (n = 2, patient nos. 9, 10) were stained with FITC-conjugated mouse-antihuman CD19 monoclonal antibodies (FMC63, Serotec, U.K.) and phycoerythrin (PE)-conjugated mouse anti-human CD138 monoclonal antibodies (B-B4, ImmunoQuality Products, Groningen, the Netherlands) and purified by fluorescence-activated cell sorting (FACS) with a MoFlo™ High-Speed sorter (Cytomation, Heidelberg, Germany). Purity of sorted cells was >98% (data not shown). Purified $CD19^+$ $CD138^-$ B cells were cultured for 7 d in the presence of irradiated (100 Gy) 3T6CD40L cells and 10 ng/ml rhIL-4 in flat-bottom 24-well plates at a concentration of 4 x 10^4 cells/well. Cells were harvested, washed twice with HBSS and re-seeded at 4 x 10^4/well and cultured for an additional 7 d in the presence of irradiated (100 Gy) untransfected 3T6 cells, 50 ng/ml rhIL-10 and 1 ng/ml rhIL-2 (Peprotech CE). End-differentiation of B cells was monitored by flow cytometric analysis on cultured cells and immunoglobulin levels were measured in culture supernatants.

FACS analysis

The following monoclonal antibodies were used for FACS analysis of cultured B cells: FITC-conjugated mouse anti-human CD19 (FMC63, Serotec), PE-conjugated mouse anti-human CD38 (T16, ImmunoQuality Products) and PE-conjugated mouse-anti-human CD138 (B-B4, Immunoquality Products). FACS analysis was performed on a Coulter Epics™ Elite flow cytometer. Data was analysed using the WINLIST software package (Verity Software House, Topsham, MN) and WINMDI shareware.

Detection of immunoglobulins in culture supernatant

Immunoglobulin levels in culture supernatant were measured with an Ig-specific enzym-linked immunosorbent-assay (ELISA). Triplicate wells of serially diluted supernatants were analysed. Maxisorp™ (NUNC) 96-wells plates were coated overnight with 10 μg/ml polyclonal rabbit anti-human IgM, IgG, IgA antibodies (DAKO, Glostrup, Denmark). Plates were washed with phosphate-buffered saline (PBS) and blocked with PBS + 1% BSA for 1 h at 37°C. Culture supernatant was added and incubated for 1 h at 37°C. Plates were washed three times with PBS + 1% BSA + 0.05% Tween 20. Bound immunoglobulins were detected with alkaline phosphatase conjugated polyclonal rabbit-anti-human Ig isotype-specific antibodies (IgM, IgG and IgA, H + L) (DAKO). Plates were developed with 2 mg/ml P-nitrophenyl (AP-substrate 104™ Sigma Chemicals) in 2-amino-2-methyl-1,3-propanediol buffer (pH = 10.25). Optical density was determined at 405 nm wavelength. Concentration of Ig was determined by comparing with standard serum curves.

Quantitative ASO-PCR

DNA was isolated from purified B cells and cultured B cells using the Qia-Amp Blood kit (Qiagen, Hilden, Germany), and quantified using Hoechst 33258 dye (Polysciences Inc. Warrington, PA) and a fluorometer (Hoeffer Instruments, Amersham-Pharmacia Biotech). DNA was serially diluted in MilliQ in squareroot 10 decrements. Quantitative limiting dilution ASO-PCR was performed essentially as described.[20] In short, the amount of amplifiable DNA was estimated using fourfold dilutions (squareroot 10^8 to squareroot 10^{11}) in a semi-nested β-globin reference PCR. Five microlitres of the DNA dilutions was subjected to a first step PCR using 1.5 mmol/l $MgCl_2$, subsequently 2 μl first-step PCR product was subjected to a second step using 5' nested oligonucleotides. For quantitative analysis of clonotypic cells, 10 μl DNA dilution was initially amplified with 30 pmol VHfamily-specific FR1 and JH21 oligonucleotide. Two microlitres of the PCR product was subjected to a nested PCR using 30 pmol patient-specific CDR1/CDR2 and CDR3 oligonucleotide. Second step PCR was performed with 2 mmol/l $MgCl_2$. PCR-products were separated on a 2% agarose gel. The most diluted sample that was positive in the above mentioned procedure was subjected together with the two lower and higher diluted samples to ASO-PCR in threefold or fourfold. The number of input cells and the number of clonotypic cells were calculated using a programme based on the single-hit Poisson model. In this programme each positive and negative PCR reaction at different dilution levels is subjected to Poisson distribution statistic.[22] The weighted mean estimate was used for Newton's iterative approximation. The maximum likelihood and minimum χ^2 were calculated as described.[23] Estimates were accepted if χ^2 values were within 95% significance level.

T(4;14)(p16;q32) IgH-MMSET fusion transcript RT-PCR

For patient no. 7, a t(4;14) specific RT-PCR was essentially performed as described.[24] In short, Iμ specific oligonucleotides (Iμ1) were combined with WHSC1/MMSET exon 6 specific oligonucleotides (ms6r). For nested PCR, 1 μl of first-round PCR product was amplified for an additional 30 cycles with nested Im oligonucleotides (Iμ2) and a WHSC1/MMSET exon 5 specific oligonucleotide (ms5r).

RESULTS

Clonotypic B cells expressing variant isotypes are present in peripheral blood of MM patients and can be detected in CD40 culture system.

T cell-depleted peripheral blood samples from 6 MM patients (no. 1 - no. 6) were analysed for the presence of clonotypic B cells expressing variant isotypes using an isotype-specific ASO-RT-PCR strategy. In three patients, clonotypic B cells expressing variant isotypes were detected. In patient 1 (IgD-secreting MM) IgG- and IgA-expressing cells harbouring the identical V(D)J rearrangements as the malignant plasma cells from the bone marrow were detected. In patient 2 (IgG-secreting MM) IgM and IgA isotype variants were detected. In patient 4 (IgG-secreting

Table I. Detection of clonotypic B cells producing variant Ig-isotypes after culturing in the CD40-system. *Analysed by ASO-RT-PCR after 14 days of culturing. † Biclonal Multiple Myeloma (Bakkus *et al*, 2000). T cell depleted peripheral blood cells were analysed by Ig-isotype specific ASO-RT-PCR before culturing and after 7 days of culturing in the CD40-system. The total number of ASO-RT-PCR positive wells for the indicated Ig-isotypes vs. the total number of wells is indicated. n.d. Not determined.

Patient	M-protein isotype	Isotype variants before culture	IgM	IgD	IgG	IgA	IgE
1	IgDλ	IgG, IgA	0/12	2/12	5/12	5/12	n.d.
2	IgGκ	IgM, IgA	12/12	n.d.	12/12	12/12	n.d.
3	IgGκ	-	0/6	n.d.	2/6	0/6	n.d.
4	IgGκ	IgA	0/16	n.d.	16/16	7/16	n.d.
5	IgAκ	-	0/6	n.d.	0/6	2/6	n.d.
6*	IgAκ + IgEκ†	-	0/10	n.d.	0/10	7/10	10/10

MM), we found clonotypic IgA transcripts (Table I). The detection of clonotypic V(D)J transcripts coupled to upstream and downstream isotypes in the peripheral blood suggests the existence of a circulating pre-Ig switch precursor cell in MM.

To determine the ability of clonotypic B cells, expressing variant Ig-isotypes, to grow and survive in the CD40 culture system, we cultured T cell-depleted PB cells from these six patients for 7 or 14 d in the presence of CD40L and IL-4, which selectively supports B-cell growth, without inducing terminal B cell differentiation.[25] FACS analysis showed that cultured B cells express CD19 but stain negatively for CD38 and CD138, indicating that plasma cells were not present in the CD40 cultures (data not shown). Additionally, RT-PCR, using CD138-specific oligonucleotides, was performed on RNA isolated from B cells before and after culturing. As depicted in figure 1A, the CD19 sorting-procedure provided only B cells without plasma cells, reflected by the absence of CD138 transcripts. Upon culture, no evidence was obtained that CD138-expressing cells were present, indicating that the cells did not differentiate to mature plasma cells. To determine whether clonotypic B cells expressing variant Ig isotypes could be detected in the CD40 system, we isolated and analysed RNA from separate wells (24-well plates) by isotype-specific ASO-RT-PCR. Ig-isotype variants that were found before culturing could readily be detected after culturing (table I).

To show that the studied clonotypic B cells belonged to the malignant clone, an RT-PCR was performed for the specific t(4;14) fusion transcript present in fresh bone marrow plasma cells from patient 7. RT-PCR demonstrated that the sorted and cultured $CD19^+/CD138^-$ B cells harbour identical translocation fusion transcripts as the malignant plasma cells in the bone marrow (Figure 1C).

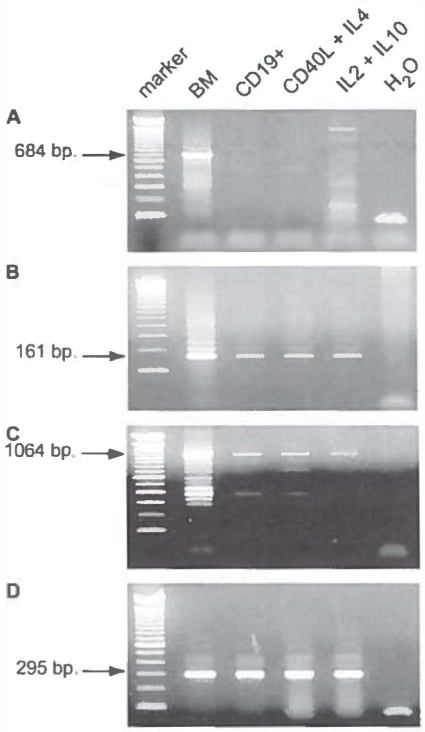

Figure 1. Clonotypic B cells are present after culture as assessed by ASO-RT-PCR and t(4;14)-specific RT-PCR. RT-PCR was performed on bone marrow mononuclear cells, flow cytometry-sorted CD19⁺ peripheral blood cells, CD40L + IL-4 stimulated B cells and IL-10 + IL-2 stimulated B cells obtained from patient 7. (A) CD138-specific oligonucleotides. (B). CDR1 and CDR3 patient-specific oligonucleotides (ASO-RT-PCR). (C) Iμ1 and MMSET exon 6-specific oligonucleotides t(4;14) fusion transcript RT-PCR. (D) β-Actin-specific oligonucleotides.

Figure 2. Detection of an IgG-expressing peripheral blood clonotypic B cell subclone. Immunoglobulin-specific ASO-RT-PCR was performed on bone marrow mononuclear cells and T-cell depleted peripheral blood cells from patient 8. An IgA-expressing isotype variant was found in the bone marrow obtained at diagnosis. T-cell depleted peripheral blood cells were cultured for 14 days in the CD40 system and subcloned in 96 wells at a concentration of 10 cells/well. Subcloned wells were analysed after 28 days of culture. One ASO-RT-PCR-positive well was identified. CDR1-Cμ, CDR1-Cγ, CDR1-Cα RT-PCR products were blotted and hybridized with a dig-tailed CDR3 oligonucleotide probe.

To confirm the presence of clonotypic B cells, we subcloned an ASO-RT-PCR positive well from an additional IgG-secreting MM patient (patient 8) after 14 d in the CD40 system. The subcloned wells were analysed after 28 d. One of the 96-screened wells was ASO-RT-PCR positive. Southern blotting showed that the subclone was positive for IgG (figure 2), whereas in bone marrow also an IgA-isotype variant was detected. This finding indicates that clonotypic B cells can be subcloned *in vitro* although the relative number of clonotypic B cells in this culture system was low.

Clonotypic B cells proliferate in response to CD40L and IL-4 stimulation.

To assess whether CD40 and IL-4 triggering has a differential proliferative effect on clonotypic B cells compared with non-clonotypic (normal) B cells, we determined the relative number of clonotypic B cells before culturing and after 7 d of culture in the CD40 system. Flow cytometry-sorted $CD19^+/CD138^-$ B cells from two patients (patients 9 and 10) were cultured. Cultures were initiated at a concentration of 4×10^4 cells/well. A strong increase of cell number was observed in both cases after culture. A median increase of 4.4-fold was noticed (data not shown). The number of clonotypic B cells after 7 d of CD40L and IL-4 stimulation was determined by limiting dilution quantitative ASO-PCR. In both patients the relative number of the clonotypic B cells was not altered after 7 days of culture (table II), implying that clonotypic B cells responded to CD40 and IL-4 triggering in a similar way as non-clonotypic B cells.

Normal peripheral blood B cells from MM patients readily differentiate into antibody-secreting cells in vitro, whereas the frequency of clonotypic B cells diminishes under identical circumstances.

To assess whether triggers involved in B-cell differentiation have a differential effect on clonotypic compared with non-clonotypic (normal) B cells, the relative number of clonotypic B cells was assessed before and after B cell differentiation *in vitro*. To this end, $CD19^+$ $CD138^-$ B cells from two patients (patients 9 and 10) were cultured for 7 d in the presence of CD40L and IL-4. This was followed by a second culture period of 7 d in the presence of untransfected 3T6 cells with IL-10 and IL-2, thereby disrupting memory B-cell formation and enhancing B-cell terminal differentiation.[26]

After the second culture period, the cell number decreased to a median of 1.4-fold. To confirm B-cell differentiation *in vitro* we determined Ig concentrations in culture supernatants after the first and the second culture period. After induction of B-cell differentiation, Ig-ELISA shows a sharp increase of IgM, IgG and IgA concentrations in the supernatant of the cultures from all patients, indicating that B cells from MM patients are capable of differentiating towards Ig-secreting cells *in vitro*. After IL-10 and IL-2 stimulation IgM showed a ninefold to 72-fold increase, IgG a 21-fold to 151-fold increase and IgA a fivefold to 12-fold increase.

The Ig concentration in the culture supernatant after the second culture period appears to be negatively correlated with the increase in cell number in the CD40L and IL-4 cultures. FACS-

Table II. Relative number of clonotypic B cells detected by limiting dilution ASO-PCR after induction of proliferation and differentiation *in vitro*. *A cell number of 160,000 (1 μg genomic DNA) was used to calculate weighted estimates.

Patient number	estimate*	95% confidence interval	Goodness-of-fit $\chi 2$
Patient 9 (IgAκ)			
before culturing	123,02	43,35 - 146,80	0,195
CD40L + IL-4	118,44	81,97 - 213,36	0,66
IL-10 + IL-2	45,10	40,78 - 84,33	2,118
Patient 10 (IgGκ)			
before culturing	16,39	9,28 - 70,12	0,698
CD40L + IL-4	15,17	8,32 - 86,46	1,618
IL-10 + IL-2	1,89	1,02 - 11,59	0,327

analysis of the cultured $CD19^+$ B cells after the second culture period shows high expression of CD38 without expression of CD138.

To assess whether clonotypic B cells were involved in differentiation, the relative number of clonotypic B cells was determined in these cultures by limiting dilution quantitative ASO-PCR. In both patients the relative number of clonotypic B cells dropped (sevenfold and twofold respectively) during the second culture period (Table II).

DISCUSSION

In this study, we showed that clonotypic B cells were present in the peripheral blood of MM patients. By Ig isotype-specific ASO-RT-PCR, we demonstrated that some of these cells express variant isotypes, which is consistent with the existence of a pre-Ig switch MM precursor cell that belongs to the malignant clone. This is especially supported by the finding that the t(4;14) transcript can be demonstrated in bone marrow plasma cells as well as in peripheral blood $CD19^+/CD138^-$ cells. The role of a circulating MM precursor cell population is a much-disputed issue. It has been suggested that the majority of circulating B cells in MM patients are clonally related to the malignant plasma cells in the bone marrow,[8,27] while others found that clonotypic B cells represented a minority of the circulating B cells.[28] We found a low frequency of clonotypic B cells in untreated MM patients, which is in concordance with a previous report by Chen & Epstein.[28]

Using the CD40 culture system, we demonstrated that circulating clonotypic B cells from untreated MM patients could be triggered to proliferate, but did not respond to B-cell differentiation triggers *in vitro*. Both before and after the CD40L and IL-4 culture step, the

same variation in isotype-expression was found. As exclusively B cells and no plasma cells were found in these cultures, the variant isotypes must be already expressed at the circulating B cell stage.

Functionally, clonotypic B cells responded to CD40-triggering in a similar fashion as non-clonotypic (normal) B cells from the same patient, reflected by an increase in the B cell number expressing the clonotypic V(D)J-transcripts. This finding was in contrast with other lymphoid malignancies. In mantle cell lymphoma, CD40L selectively induces proliferation in an Ig light-chain-restricted manner,[29] which can further be enhanced by addition of IL-10.[30] However, other aspects of the proliferation and differentiation program seem to be hampered in the clonotypic B cells. Evidence was obtained that these cells do not have the potential to differentiate in response to IL-10 and IL-2 stimulation.

Somatic mutations present in the V(D)J rearrangement indicate that clonotypic B cells are of memory B-cell or plasmablast origin. The cessation of CD40L stimulation might have differential effects on normal B cells *vs* clonotypic B cells because of their activation status. This was also supported by the finding that memory B cells triggered by CD40-stimulation demonstrate an increased susceptibility for B-cell receptor-mediated apoptosis *in vitro*.[31] Furthermore, the timing of IL-10 is crucial for the biological effect on B cells. Addition of IL-10 to preactivated B cells has an antiapoptotic effect, whereas IL-10 enhanced the apoptosis during B-cell activation.[32] It might be that IL-10 engenders cell death rather than differentiation of myeloma clonotypic B cells. Previous reports have shown that IL-10 acts as a growth factor for MM cells, which is mediated via Oncostatin M (OM) in a gp130-dependent manner.[33] However, IL-10 does not have an effect on rate of Ig secretion by MM cells, indicating that IL-10 does not act as an differentiation factor for MM plasma cells.[34] Our data suggest that IL-10 is neither a differentiation nor a survival factor for circulating myeloma clonotypic B cells.

If clonotypic B cells are to play a role in the aetiology of MM, other triggers should suffice end-differentiation and survival of clonotypic B cells. For example, CD27/CD70 and CD134/CD134L (OX40/OX40L) interactions are implicated in promoting plasma cell differentiation.[35,36] In conclusion, the ability of clonotypic B cells to undergo B cell differentiation *in vitro* is hampered. Analysis of the functional aberrant behaviour of clonotypic B cells both *in vitro* and *in vivo* can possibly clarify their role in the aetiology of MM.

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chapter six

CD27 is heterogeneously expressed in multiple myeloma; low CD27 expression in patients with high risk disease

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SUMMARY

Expression of CD27 on malignant plasma cells (PC) ($CD138^+$ $CD38^{++}$) was analysed in a cross-sectional study of bone marrow (BM) samples from multiple myeloma (MM) patients (n = 28), patients suffering from monoclonal gammopathy of undetermined significance (MGUS) (n = 6) and BM PC from healthy donors (n = 4). MM PC expressed CD27 with a variable lower intensity pattern compared with the consistent high expression in MGUS and healthy donors. MM patients in complete clinical remission displayed a higher percentage of $CD27^+$ PC compared with patients at diagnosis, relapse or in partial remission. In MM, loss of CD27 correlated with loss of CD19 (R^2 = 0.4, P < 0.0001). Human MM cell lines (n = 9) did not express CD27 whereas *de novo* plasma cell leukaemia (PCL) (n = 3) had a high expression. Re-analysis of a cDNA microarray data set, generated from newly diagnosed MM patients (n = 74) demonstrated that the MM subgroup with the highest prevalence of poor prognostic factors had the lowest CD27 mRNA expression. Fluorescence-activated cell sorting and allele-specific oligonucleotide polymerase chain reaction showed that both $CD27^+$ and $CD27^-$ PC subpopulations in MM can belong to the clonal disorder. In conclusion, CD27 is heterogeneously expressed on MM PC and loss of CD27 expression might have prognostic value in MM.

INTRODUCTION

Multiple Myeloma (MM) is characterized by a clonal accumulation of malignant plasma cells in the bone marrow. The plasma cells are phenotypically characterized by a strong expression of CD38 and CD138 (syndecan-1)[1], but can display an aberrant phenotype compared with normal plasma cells. Asynchronous expression has been reported for CD56 (NCAM)[2] that is expressed in the majority of MM patients; CD117 (c-kit), which is expressed in approximately one third of myeloma patients[3] and CD28 and CD86 that are associated with extramedullary expansion of the tumour clone.[4] Mature myeloma plasma cells usually lack expression of CD19.[5] The importance of these phenotypic changes in relation to progression is largely unclear.

CD27 is a 110-kDa homodimeric transmembrane glycoprotein of the tumour necrosis factor receptor (TNFR) family.[6] CD27 is expressed by a subset of B cells and by the majority of peripheral T cells.[7-9] CD27-expressing peripheral blood B cells express somatically mutated surface immunoglobulin (Ig) receptors, identifying them as memory B cells.[10-12] The natural ligand for CD27 is CD70, a member of the TNF family. CD70 is expressed on activated B and T cells,[13-17] and the CD27/CD70 interaction is implicated in B-cell differentiation and survival.[18-21] Normal plasma cells isolated from tonsils and the gut lamina propria, and *in vitro* generated plasma cells strongly express CD27.[22,23]

In the malignant counterpart, CD27 is expressed and released as soluble CD27 by B cell malignancies representing mature B cells, including B cell chronic lymphatic leukaemia (B-CLL). In two previous studies it was suggested that CD27 is not expressed on MM plasma cells.[18,24] However, both studies analysed only a limited number of MM patients. In this study, we present a comprehensive analysis of CD27 expression in a cross-sectional MM patient group (n = 28). We show that CD27 can be expressed on malignant plasma cells in MM but with a variable expression pattern. Importantly, plasma cells from MM patients in complete clinical remission display a significantly higher CD27 expression compared with those obtained from newly diagnosed, relapsed and refractory MM patients. In a previous study, it was shown that gene profiling of newly diagnosed MM patients identified hierarchical clustered MM subgroups, which are associated with the prevalence of established adverse prognostic factors.[25] By re-analysis of this data set, we demonstrated that CD27 was differentially expressed in hierarchical clustered MM subgroups. MM patients displaying the lowest CD27 expression were all clustered in the high-risk patients groups, of which the mRNA expression profile closely resembles that of human MM cell lines. Additionally, CD27 expression was studied in plasma cells from healthy donors, from monoclonal gammopathy of undetermined significance (MGUS) patients and patients suffering from *de novo* plasma cell leukaemia (PCL) and it was demonstrated that in contrast to MM, plasma cells from healthy donors, MGUS and PCL patients show a homogeneous high CD27 expression.

MATERIALS & METHODS

Patient material

For flow-cytometric analysis, bone marrow cells from iliac crest were obtained from MM patients (n = 28) at various stages of treatment and disease. Six samples were obtained at presentation, 12 samples from relapsed patients, four samples from patients in partial remission and six samples from patients who achieved a complete remission based on cytological criteria. Furthermore, bone marrow cells were obtained from newly diagnosed plasma cell leukaemia (PCL) patients (n = 3), monoclonal gammopathy of undetermined significance (MGUS) patients (n = 6), solitary plasmacytoma patients (n = 2), one cold agglutination IgM syndrome patient, one AL-amyloidosis patient and from healthy donors (n = 4). For microarray mRNA expression analysis, anti-CD138 (Miltenyi-Biotec, CA, USA) plasma cell purification from 32 normal healthy donors and 74 patients with untreated newly diagnosed MM has been described.[25] The mononuclear cell fraction from aspirates was separated by a standard Ficoll-Hypaque gradient centrifugation (Amersham-Pharmacia Biotech). Tonsils were obtained from 11 patients undergoing tonsillectomy for chronic tonsillitis. B-cell isolation from seven tonsil preparations was performed using directly conjugated mouse anti-human CD19 monoclonal antibody, plasma cells were purified as described above (Miltenyi-Biotec). All samples were obtained after informed consent. MM and PCL patients were staged according to the Salmon and Durie criteria.[26]

Cell lines

The human MM cell lines JJN3, OPM-1, U266, RPMI8226, LB84-1, LP-1, EJM, MM-S1, Karpas 707 (courtesy of Dr. MHC Bakkus) were maintained in supplemented Roswell Park Memorial Institute (RPMI)-1640 medium with 10% fetal calf serum (FCS, Life Technologies, Gaitherburg, MD, USA).

Monoclonal antibodies

The following mouse-anti-human monoclonal antibodies were used in this study: peridine chlorophyll protein (PerCP)-conjugated anti-CD19 (SJ25-C1, Becton & Dickinson, Mountain View, CA, USA), allophycocyanin (APC)-conjugated anti-CD38 (T16, ImmunoQuality Products, Groningen, the Netherlands), fluorescein isothiocyanate (FITC)-conjugated anti-CD138, phycoerythrine (PE)-conjugated anti-CD138, APC-conjugated anti-CD138 (B-B4, ImmunoQuality Products), PE-conjugated anti-CD27 (MT-271, BD Pharmingen, San Diego, CA, USA), FITC-conjugated anti-CD27 (CLB27/1, Central Laboratory of The Netherlands Red Cross, Amsterdam, The Netherlands), biotinylated anti-CD27 (O323, E-Bioscience, San Diego, CA, USA). FITC-conjugated anti-Kappa and anti-Lambda light chain (DAKOpatts, Glostrup, Denmark). Isotype- & fluorochrome-matched controls were included in all experiments.

Flow cytometry analysis

Four-colour staining procedures were performed on freshly obtained bone marrow cells; red cells were lysed using FACS-brand™ lysing solution (Becton & Dickinson, CA, USA). Intracellular staining of Ig-light chains was performed using the Fix and Perm intracellular staining kit according to the manufacturer's instructions (an der Grub, Kaumberg, Austria). Cryopreserved mononuclear cells were thawed in newborn calf serum (NCS, Life Technologies). Thawed bone marrow cells were treated with DNase I (0,25 mg/mL), $MgSO_4$ and heparin (125U/mL); cells were washed prior to staining. The staining of nucleated cells was determined by gating, based on forward and side scatter (FSC/SSC) properties. Cell debris and red blood cells were excluded by FSC/SSC gating. A minimum of 40,000 events were measured using a FACSCalibur apparatus (Becton & Dickinson). Four-colour staining was analysed using the WINLIST software package (Verity Software house Inc, ME, USA) and the FLOWJO package (TreeStar, Stanford, CA, USA). CD27 expression was determined within the $CD138^+$ $CD38^{++}$ plasma cell gate. The percentage of positive cells was calculated by subtracting isotype-control histograms from the CD27 histograms using the enhanced normalisation subtraction (ENS) protocol from the WINLIST software package. Mean fluorescence intensities were calculated for CD27 and CD19. Normalization was performed by calibrating the flow-cytometer using Calibrite™ beads (Becton & Dickinson).

Re-analysis of cDNA microarray dataset

Detailed protocols for cell purification, cDNA synthesis, cRNA preparation, and hybridization to the Affymetrix HuGeneFL GeneChip microarray have been described in a previous study.[25] The natural log of the average difference of the *TNFRSF7* (CD27) gene expression was determined in this data set. The natural log of the average difference was used in a one-way analysis of variance (ANOVA) using separate means for each of the groups, B cells (BC), tonsillar plasma cells (TPC), bone marrow plasma cells (BPC) and MM subgroups, and compared them individually.

Flow cytometry sorting

$CD138^+$ $CD38^{++}$ $CD27^+$ and $CD138^+$ $CD38^{++}$ $CD27^-$ plasma cell populations were sorted from the bone marrow from a MM patient using a MoFlo high-speed sorting device (Cytomation, Heidelberg, Germany). Purity of sorted populations was > 98%, as assessed by re-analysis.

Reverse transcription polymerase chain reaction (RT-PCR) and cloning of patient-specific Ig VH genes

Total RNA was isolated from 1 x 10^6 bone marrow mononuclear cells, sorted plasma cells and human MM cell lines using Trizol™ reagent (Life Technologies) according to the manufacturer's instructions. Approximately 1 μg of RNA was primed with 165 ng random hexamers (Amersham-

Pharmacia Biotech) and murine Moloney leukaemia virus reverse transcriptase (SuperscriptII™, Life Technologies) in a volume of 20 μl, containing 50 mmol/l KCl, 20 mmol/l Tris-HCl (pH 8.4), 2.5 mmol/l $MgCl_2$, 1 mmol/l dithiothreitol and 0.5 mmol/l of each deoxynucleotide triphosphate (dNTP), for 50 minutes at 42°C. The reaction was stopped by enzyme inactivation for 5 min at 95°C. cDNA integrity was confirmed by β-Actin RT-PCR (β-Actin forward: 5'-TCA CCC ACA CTG TGC CCA TCT ACG A-3'; β-Actin backward: 5'-CAG CGG AAC CGC TCA TTG CCA ATG G-3'). The Ig VH genes expressed by the malignant plasma cells were amplified using VH family-specific Framework 1 (FR1) and Ig-constant region-specific oligonucleotides as described.[27] cDNA (0.5 μl) was amplified in a 25 μl PCR reaction containing 20 mmol/l Tris-HCl (pH 8.4); 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, with 15 pmol/l of each primer, and 1.25 U Taq DNA polymerase (Life Technologies); all PCR reactions were performed at an annealing temperature of 60°C for 30 amplification cycles. PCR products were separated on LE agarose gels (Seakem, FMC Bioproducts, Rockland, MN, USA) and visualized by ethidium bromide staining and ultra violet illumination. PCR products were cloned directly into the pCR2.1TOPO™ vector (Invitrogen, Leek, the Netherlands) according to the manufacturer's instructions. Positive colonies were identified by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranisode (X-gal) blue/white screening and EcoRI restriction enzyme analysis. Plasmids were isolated with the High-Pure plasmid isolation kit (Roche Molecular Biochemicals, Mannheim, Germany). At least eight clones were partially sequenced using only the dideoxy thymidine triphosphates (ddTTP) ('T-tracks') to identify clones that contained the dominant tumour-derived IgH sequence. Dominant clones were then cycle sequenced with the complete set of ddNTPs using FITC-labelled plasmid-specific oligonucleotides (M13forward and M13reverse). Sequencing gels were analysed using the ALFwin automated sequencing device (Amersham-Pharmacia Biotech). DNA-sequences were compared with the ImMunoGeneTics (IMGT) sequence database using DNAplot software (http://imgt.cines.fr:8104) and submitted to the European Molecular Biology Laboratory (EMBL) database (available under accession number AJ316217). Allele-specific oligonucleotides (ASO) were designed for the hypervariable complimentarity determining regions 1 and 3 (CDR1 and CDR3). The CDR1 oligonucleotide was designed to bind a somatic mutation at the 3' end. The CDR3 oligonucleotide was chosen in the N-region from the D-JH junction. Oligonucleotide specificity was confirmed by RT-PCR on bone marrow from healthy donors and other MM patients. CD27 RT-PCR was performed using the conditions detailed above (CD27 forward: 5'-CCA GCT TGG AGG TGC TAA CT-3'; CD27 backward: 5'-TGC AGG CTC CAC AGG ACT T-3').

Statistical analysis

Spearman's nonparametric test was used to calculate the correlation coefficients. The Mann-Whitney *U*-test was used to determine any significant differences between groups.

RESULTS

Multiple Myeloma plasma cells are characterized by a heterogeneous expression of CD27.

The percentage of CD27 expressing $CD138^{+}$ $CD38^{++}$ plasma cells was analysed in a cross-sectional study of bone marrow samples from MM patients in various stages of the disease and treatment (n = 28). The percentage of CD27 expressing bone marrow plasma cells from MM patients showed a marked heterogeneity (Figs 1A-C). MGUS patients (n = 6) had a significantly higher percentage of CD27-expressing plasma cells compared with MM patients (mean 93.48 ± 8.49 vs. 67.02 ± 30.2, P = 0.0154) (Figs 1D and 2). Plasma cells obtained from healthy donors (n = 4) (Figure 1F), PCL (n = 3) (Figure 1E), solitary plasmacytoma (n = 2), cold agglutination IgM syndrome (n = 1) and AL amyloidosis (n = 1) patients all showed homogeneous high CD27 expression (> 98%). A previous study has demonstrated that, in contrast with plasma cells from healthy volunteers, plasma cells from MM patients showed loss of CD19 expression.[5] Analysis of mean fluorescence intensities showed that CD27 downmodulation was associated with CD19 downmodulation (R^2 = 0.4, P < 0.0001) (Figs 3A and B), indicating that CD27 is specifically lost from $CD19^{-}$ plasma cells in MM.

In addition, all human multiple myeloma cell lines tested (JJN3, OPM-1, U266, RPMI8226, LB84-1, LP-1, EJM, MM-S1 and Karpas 707) were negative for CD27 by flow cytometry and CD27-specific RT-PCR (Figs 1G-H). These MM cell lines were mostly derived from patients with progressive disease and, in most cases, reflect the extramedullary locations of myeloma.[28-35] To exclude the possibility that the use of different monoclonal antibodies was responsible for the discrepancy of our results compared with those of earlier reports[18,24], several CD27-specific mouse anti-human monoclonal antibody clones (M-T271, CLB27/1, O323) were tested on fresh bone marrow samples from 20 MM patients. All monoclonal antibodies yielded similar results (data not shown).

Downmodulation of CD27 mRNA expression is specific for MM plasma cells and is associated with high-risk disease.

Patients who achieved a complete clinical remission upon treatment were characterized by a significantly higher percentage of CD27-expressing plasma cells. (98.5 ± 1.8%) compared with newly diagnosed and relapsed patients (mean 67.0 ± 30.2%, P = 0.0022) (Fig 2). To further assess the significance of CD27 expression, seven additional groups were analysed by cDNA microarray; tonsil B cells (BC; n = 7), tonsil plasma cells (TPC; n = 11), bone marrow plasma cells (BPC, n = 32), and gene expression profile MM patient subgroups (MM1, n = 20; MM2, n = 21; MM3, n = 15; and MM4 n = 18) defined by hierarchical clustering as described.[25] By comparing 5,483 genes, patients were grouped according to gene expression profile in which MM1 expression pattern was similar to normal plasma cells and MGUS, whereas MM4 was similar to the MM cell lines. Clinical parameters have been compared among clustered MM subgroups. Clinical parameters linked to poor prognosis (abnormal cytogenetics, high serum β2 microglobulin,

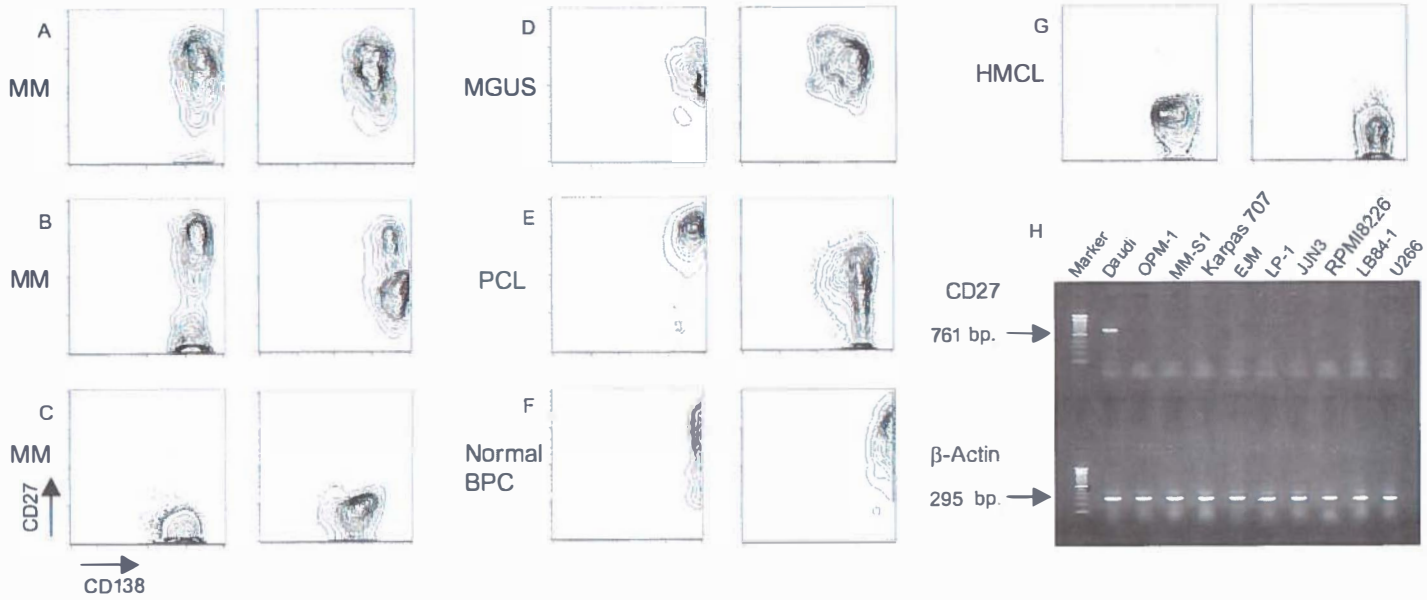

Figure 1. CD27 expression on plasma cells. CD27 expression was assessed by four-colour flow cytometry and RT-PCR. CD27 expression was determined within the CD138^{+} CD38^{++} plasma cell gate; two representative patients for each subgroup are shown: (A) multiple myeloma patients in complete clinical remission. (B) newly diagnosed multiple myeloma patients. (C) multiple myeloma patients with progressive disease, (D) MGUS patients, (E) PCL patients, (F) normal donor bone marrow plasma cells, (G) human myeloma cell lines LB84-1 and JJN3, and (H) CD27-specific RT-PCR on human myeloma cell lines. Burkitt's lymphoma cell line Daudi was used as a positive control. β-actin RT-PCR was performed to confirm cDNA integrity.

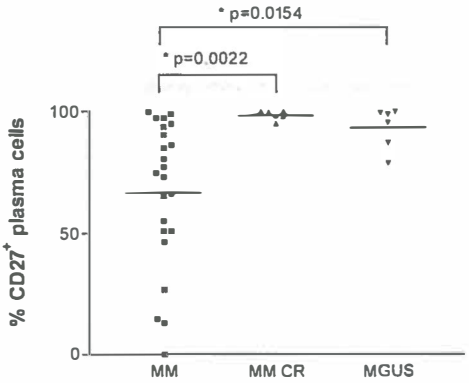

Figure 2. CD27 expression on plasma cells from multiple myeloma and MGUS patients. The percentage of CD27-positive plasma cells (CD138$^+$ CD38^{++} gate) was determined by enhanced normalization substraction. Newly diagnosed, relapsed and patients with progressive MM were compared with patients who achieved complete clinical remission (MM CR), and MGUS patients. The Mann-Whitney *U*-test was used to determine significance of differences.

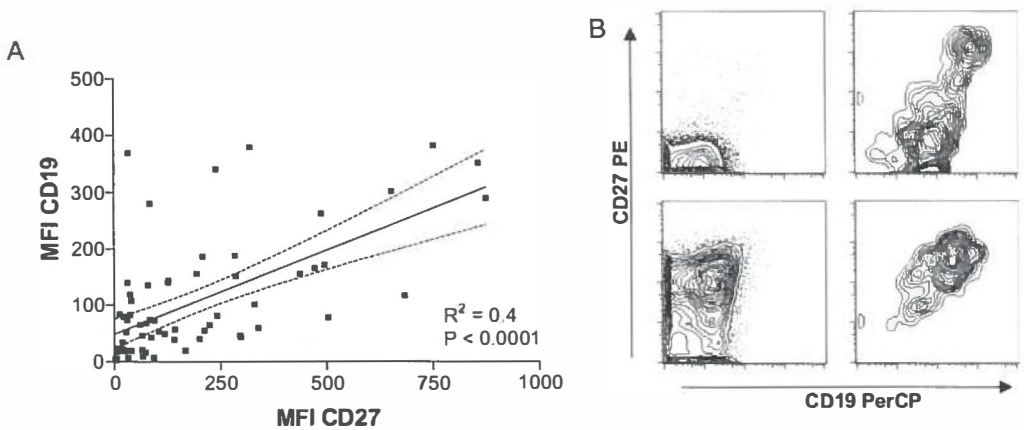

Figure 3. Loss of CD27 expression on plasma cells is associated with loss of CD19 expression. (A) Mean fluorescence intensities (MFI) for CD27 and CD19 were determined within the CD138$^+$ CD38^{++} plasma cell gate. MFI values for CD27 were plotted against MFI values for CD19. Each black square represents an individual patient; dashed lines represent 95% confidence intervals. (B) CD27 and CD19 expression on plasma cells (CD138$^+$ CD38^{++}) from MM patients was determined by four-colour flow cytometry. Four patients are depicted, demonstrating the association between CD27 and CD19 expression.

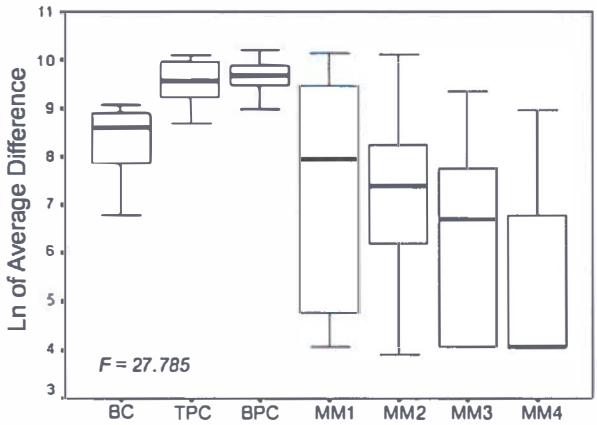

Figure 4. Box plot of *F*-values. Expression of CD27 was evaluated in seven groups. B cells (BC; $n = 7$), tonsil plasma cells (TPC; $n = 11$), bone marrow plasma cells (BPC; $n = 32$), and gene expression-defined multiple myeloma subgroups (MM1, $n = 20$; MM2, $n = 21$; MM3, $n = 15$; MM4, $n = 18$) are distributed along the *x*-axis. The natural log transformed average difference (AD) is plotted on the *y*-axis. The one-way ANOVA *F*-value for the *TNFRSF7* is presented. An *F*-value greater than 2.99 indicates a higher likelihood that a gene is variable among the groups ($P < 0.0001$). Consistent with the AD, the absolute call (AC) indicated that the *TNFRSF7* gene was present in all BC, TPC and BPC samples, but was absent in a significant portion of MM subgroups and showed highly significant loss in MM4 ($P < 0.01$).

chromosome 13 deletions) were most prevalent in MM4.[25] In this study, we compared *TNFRS7* (CD27) gene expression in these hierarchical clustered MM subgroups and normal cells using this data set. All MM cases had lower *TNFRSF7* (CD27) gene expression than their normal BPC counterparts. A gradual decrease of CD27 mRNA expression could be observed through MM subgroups 1-4, where the MM4 subgroup demonstrated the lowest level of CD27 expression (Fig 4).

This would appear to contrast with the homogeneous high expression of CD27 on *de novo* PCL that is generally associated with a higher incidence of poor prognostic factors and, consequently, a more aggressive clinical course. The PCL patients presented in this study showed the typical clinical behaviour of poor prognosis PCL.

Both CD27$^+$ and CD27$^-$ plasma cells belong to the clonal disorder in MM.

In 17/28 patient samples, distinct CD27$^+$ and CD27$^-$ plasma cell subpopulations could be distinguished (figure 1B). Sorted CD27$^+$ and CD27$^-$ plasma cells (CD138$^+$ CD38^{++}) from the bone marrow of a newly diagnosed MM patient ($n = 1$) express the malignant plasma cell V(D)J rearrangement as determined by CDR1-CDR3 RT-PCR. Restricted Igκ light chain expression was observed in both CD27$^+$ and CD27$^-$ plasma cell populations (Fig 5).

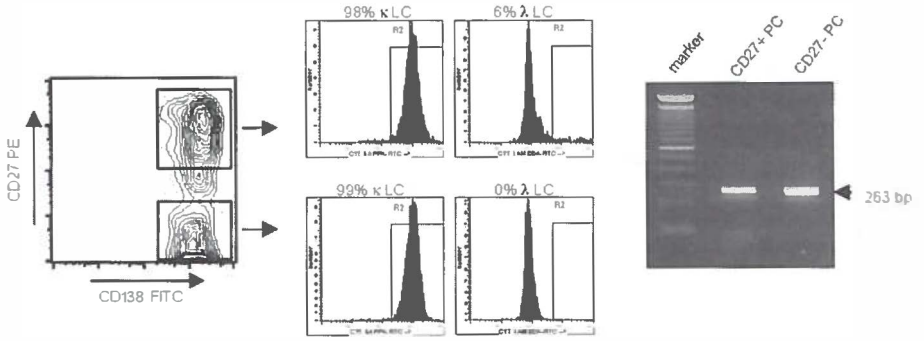

Figure 5. CD27-positive and CD27-negative plasma cells both belong to the clonal disorder as determined by Ig light chain expression and ASO-RT-PCR. Bone marrow mononuclear cells from an IgGκ-expressing MM patient at presentation were sorted to obtain $CD138^{+}CD38^{++}CD27^{+}$ and $CD138^{+}CD38^{++}CD27^{-}$ subpopulations. The purity of sorted cells was >98% as assessed by re-analysis. Immunoglobulin κ and λ light chain expression was determined by intracellular staining. ASO-RT-PCR was performed on sorted populations using CDR1 and CDR3-specific oligonucleotides.

DISCUSSION

The present study demonstrated that CD27 is expressed on malignant plasma cells in a heterogeneous fashion. MM patients in complete clinical remission showed a significantly higher percentage of CD27-expressing plasma cells compared with MM patients at diagnosis and at relapse. This heterogeneity might be responsible for the discrepancy between our results and those of earlier reports,[18,24] in which only a limited number of patients were analysed. Our data suggest that progression/relapse of MM is associated with loss of CD27 expression and that clinical response, which is associated with the reappearance of normal plasma cells, can be monitored by assessment of CD27 expression on plasma cells.

Bone marrow plasma cells from MGUS patients showed a homogeneous high expression of CD27, which was analogous with the CD27 expression on normal plasma cells. This is in agreement with the initial benign nature of this disease. Of interest is whether progression of MGUS to overt MM is associated with loss of CD27 expression. Loss or aberrant expression of surface markers has been described for MM. For instance, normal plasma cells have a phenotype of $CD19^{+}/CD56^{-}$ whereas malignant plasma cells are $CD19^{-}/CD56^{+}$.[5,36,37] A previous study suggests that loss of CD19 is involved in tumour progression, as enforced overexpression of CD19 in human myeloma cell lines leads to growth inhibition and reduced tumourigenicity *in vivo*.[38] Analysis of mean fluorescence intensities (MFI) demonstrated a significant correlation between loss of CD27 expression and loss of CD19 expression. This is further supported by the lack of both CD19 and CD27 expression on the human myeloma cell lines we tested (data not shown). Furthermore, $CD27^{+}$ plasma cells displayed a lower FSC profile than $CD27^{-}$ plasma cells (data

not shown), which is characteristic of more immature plasma cells. Although the $CD27^+$ plasma cell population apparently includes normal plasma cells, ASO-PCR analysis demonstrated that this population can also harbour clonotypic cells, suggesting that $CD27^+$ and $CD27^-$ plasma cell populations both can be incorporated into to the clonal disorder.

A previous study demonstrated by cDNA microarray analysis that *TNFRSF7* (CD27) gene expression was lower in MM plasma cells compared with normal plasma cells present in bone marrow and tonsil, again indicating that loss of CD27 is specific for MM. In this study, we compared hierarchical clustered MM subgroups and show that the CD27 expression was lowest in the MM4 subgroup. This group includes patients with poor prognostic factors (e.g. high β2-microglobulin and abnormal cytogenetics), supporting the hypothesis that loss of CD27 is associated with a more aggressive disease. However, this view is not in line with the results in *de novo* PCL where a homogeneous high expression CD27 was observed, in spite of a more aggressive clinical behaviour. It has been suggested that *de novo* PCL represents a different clinical entity that can be distinguished from MM on basis of immunophenotyping and chromosomal abnormalities.[39] Expression of CD27 might be regarded as an additional parameter that differentiates PCL from MM.

Distinct $CD27^+$ and $CD27^-$ plasma cell populations were present in the bone marrow of many MM patients. It is conceivable that the reduced expression of CD27 on a subpopulation of plasma cells might be related to clonal selection to a more aggressive subtype, as both the $CD27^+$ and $CD27^-$ populations are found in the malignant clone. This was supported by the results of the human MM cell lines. All tested cell lines lack CD27 at the mRNA and protein level, and were mostly derived from MM patients with progressive disease.[28-35]

A recent study has demonstrated that CD27 expression can be induced from purified normal $CD27^-$ naive peripheral blood B cells by CD40 ligation.[40] In contrast, CD40 ligation was not capable of inducing CD27 expression on the CD40 expressing myeloma cell line U266 and the Epstein-Barr virus-transformed lymphoblastoid cell line ARH-77 (data not shown), indicating that loss of CD27 expression is an intrinsic feature of MM plasma cells and probably not due to the absence of appropriate co-stimulatory signals. Further study into the regulation of CD27 and its association with progression could provide insight into the role of CD27 in the biological and clinical aspects of MM.

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chapter seven

CD27-triggering on primary plasma cell leukaemia cells has anti-apoptotic effects involving mitogen activated protein kinases

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SUMMARY

Primary plasma cell leukaemia (PCL) is a rare plasma cell malignancy, which is related to multiple myeloma (MM) and is characterized by a poor prognosis. In a previous study we demonstrated that PCL plasma cells display a high expression of CD27, in contrast to MM plasma cells. In this study we set out to assess the functional properties of CD27 expressed on PCL plasma cells by triggering with its ligand CD70. Using CD27-expressing purified plasma cells from a PCL patient we demonstrated that CD27-triggering modestly inhibited spontaneous and dexamethasone-induced apoptosis. By *in vitro* stimulation and Western blotting we showed that activation of p38 and extracellular-regulated kinase 1/2 (ERK1/2) mitogen activated protein kinases (MAPK) was associated with CD27-mediated signal transduction. Specific inhibition of p38 and ERK1/2 MAPK abolished the anti-apoptotic effects of CD27-triggering. Interestingly, simultaneous inhibition of p38 and ERK1/2 strongly sensitized PCL cells for dexamethasone-induced apoptosis. Finally, in dexamethasone treated PCL cells, CD27-triggering was associated with persistent DNA-binding activity of Activator Protein 1 (AP-1) but not of Nuclear Factor-κB (NF-κB). These findings suggest that in primary PCL specific anti-apoptotic pathways exist that might provide novel therapeutic targets.

INTRODUCTION

Primary plasma cell leukaemia (PCL) is a disease entity that is characterized by a clonal expansion of malignant plasma cells primarily in the peripheral blood (PB), but also in the bone marrow (BM).[1,2] PCL is believed to be closely related to multiple myeloma (MM). In the initial phase of MM the malignant plasma cells specifically reside in the BM. However, in end-stage disease MM, malignant plasma cells can grow independently of the bone marrow microenvironment and acquire leukaemic characteristics (secondary PCL).[3] Several studies suggest that secondary PCL in MM differs from primary or *de novo* PCL regarding immunophenotype and genetic alterations.[4-9] Importantly, compared to MM patients, primary PCL patients have a more aggressive disease history. Primary PCL is associated with poor prognostic features and a dismal clinical outcome.[3,6,10]

In a previous study we demonstrated that malignant plasma cells in PCL are characterized by a homogeneous high expression of CD27, whereas malignant plasma cells in end-stage MM are characterized by a loss of CD27 expression.[11] Additionally, we demonstrated that MM cell lines, which are mostly derived from MM patients in end-stage disease or with secondary PCL, do not express CD27 at the mRNA and protein level. CD27 is a homodimeric transmembrane glycoprotein of the tumour necrosis factor receptor family (TNFRII),[12] and is expressed on the majority of peripheral T cells and on a subset of B cells.[13-15] On B cells, CD27 can be regarded as a memory B-cell marker, as has been shown by the expression of somatically mutated immunoglobulin (Ig) receptors on CD27-positive peripheral blood B cells.[16-18] Functionally, CD27 acts as co-stimulatory molecule in plasma cell differentiation.[19] Upon triggering with its natural ligand CD70, CD27-expressing B cells rapidly differentiate into plasma cells in the presence of soluble co-stimulatory factors like interleukin-10 and interleukin-2.[20,21] It has been suggested that CD27-triggering counteracts the apoptotic effects of interleukin-10, enabling end-stage differentiation of memory B cells.[20] CD27 is strongly expressed on bone marrow plasma cells from healthy individuals, plasma cells present in the gut lamina propria and *in vitro* generated plasma cells.[22,23]

In previous studies it has been shown that upon CD27-triggering, the TNF receptor associated factors (TRAFs) 2 and 5 are recruited, resulting in the activation of nuclear factor κB (NF-κB) and/or Janus N-terminal kinase (JNK),[24-26] suggesting anti-apoptotic properties of CD27-triggering. However, the functionality and signal transduction pathway characteristics of CD27 on plasma cells from healthy individuals and primary PCL patients are unknown.

In this study we demonstrate that CD27 triggering on PCL plasma cells has anti-apoptotic properties, involving p38 and ERK1/2 MAPK activation. Furthermore, inhibition of p38 and ERK1/2 sensitised PCL for dexamethasone-induced apoptosis, providing an attractive target for novel therapeutic strategies.

MATERIALS & METHODS

In vitro CD27-triggering of PCL cells

By flow cytometry, $CD138^+$ $CD27^+$ plasma cells were purified from peripheral blood obtained from a previously treated *de novo* PCL patient with progressive disease (n = 1). Plasma cells were cultured overnight in supplemented Roswell Park Memorial Institute (RPMI)-1640 medium with 10% fetal calf serum (FCS), at 37°C, 5% CO_2. After overnight culture, dead cells and cell debris were removed by standard Ficoll-Hypaque gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden). For apoptosis and proliferation assays, 2×10^5 plasma cells were cultured for 24 hours in the presence of 2×10^5 paraformaldehyde-fixed CD70/300-19 or wild/300-19 (kind gifts from Dr. K. Agematsu) per well in round-bottom 96-well plates (Nalge Nunc International, Roskilde, Denmark). For apoptosis assays, stimulations were performed in quadruplicate, and experiments were repeated three times. For Electrophoretic Mobility Shift Assays (EMSA) and Western blotting analysis, 3×10^6 purified plasma cells were cultured in the presence of 3×10^6 paraformaldehyde-fixed CD70/300-19 or wild/300-19, in 24-well plates. CD70/300-19 is a mouse pre-B cell line stably transfected with human CD70 cDNA and is characterized by a constitutive high CD70 expression,[20] wild/300-19 is the parental cell line. Cyclodextrin-encapsulated dexamethasone (Sigma Chemicals, St. Louis, MO, USA) was added at final concentration of 0.1 μM. The p38 MAPK competitive inhibitor SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) 1H-imidazole; SmithKline Beecham Pharmaceuticals, King of Prussia, PA, USA) was added at a final concentration of 1.0 μM. The ERK1/2 inhibitor U0126 (1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene; New England Biolabs, Beverly, MA, USA) was added at a final concentration of 2.0 μM. In some experiments polyclonal goat-anti-human IL-6 serum (kind gift by dr. L. van Aarden) was added at a final dilution of 1:500.

Apoptosis assays

Apoptosis of cultured PCL cells was determined by Annexin-V binding and intracellular staining for active (cleaved) caspase-3 after 24 hours of stimulation. For Annexin-V binding, cells were washed in Annexin-V binding buffer (2.5 mmol/l $CaCl_2$, 20 mmol/l HEPES pH = 7.4, 140 mmol/l NaCl) and stained with fluorescein isothiocyanate (FITC)-conjugated Annexin-V and allophycocyanin (APC)-conjugated mouse anti-human CD38 monoclonal antibody (Immunoquality Products, Groningen, the Netherlands). After addition of 10 μg/mL propidium iodide (PI), Annexin-V binding was assessed within the $CD38^{++}$ PI^- gate on a Coulter Epics-Elite flow cytometer with enhanced system performance upgrade (Coulter, Hialeah, FL, USA). For intracellular active caspase-3 staining, cells were washed twice in ice-cold phosphate-buffered saline (PBS), fixed with Cytofix/Cytoperm™ solution (BD Pharmingen, San Diego, CA, USA) at 4°C, washed with Perm/Wash™ buffer (BD Pharmingen) and stained with APC-conjugated mouse anti-human CD38 monoclonal antibody (Immunoquality Products) and FITC-conjugated rabbit

anti-active caspase-3 monoclonal antibody according to the manufacturers instructions (BD Pharmingen). Intracellular active caspase-3 staining was determined within the $CD38^{++}$ gate. The stainings were performed in quadruplicate. All apoptosis assays were repeated 3 times. Results are given as means ± standard deviations.

Proliferation assay

PCL cells were adjusted to 10^7 cells per ml in Hank's balanced salt solution (HBSS) and labelled with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene, OR, USA) in dimethylsulfoxide (DMSO, Sigma Chemicals) at a final concentration of 0.5 μM. Cells were incubated for 10 minutes at room temperature. An equal volume of heat inactivated New-born Calf Serum (NCS, Life Technologies) was added to quench unbound CFSE. Labelled cells were washed twice in RPMI 1640 + 10% FCS. Labelled cells were stimulated as described above. After stimulation cells were stained with APC-conjugated mouse anti-human CD38 monoclonal antibody and PI. Proliferation was determined by assessment of CFSE fluorescence within the $CD38^{++}$ PI^- gate.

Western blotting

At indicated timepoints cells were harvested, washed with ice-cold PBS and lysed in 50 μl lysis-buffer containing 2% sodium dodecyl sulphate (SDS), 10% glycerol, 2% β-mercaptoethanol, 60 mmol/l Tris-HCl (pH = 6.8) and bromophenol-blue. Cell extracts were boiled for 5 min and were stored at -80°C until used. Samples were boiled shortly before loading. Fifteen microlitres of cell extracts were resolved on 12.5% SDS-polyacrylamide gel and blotted onto polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). Phosphorylation of p38 MAPK and ERK1/2 was analysed by immunoblotting with rabbit anti-human phospho-p38 MAPK (Cell Signaling Technology, Beverly, MA, USA) and mouse anti-human phospho p42/p44 ERK1/2 (New England Biolabs, Beverly, MA, USA), membranes were subsequently washed with PBS + 0.1% Tween-20 and incubated with the appropriate peroxidase-conjugated secondary swine anti-rabbit or goat anti-mouse antibodies (DAKO, Glostrup, Denmark). Immunodetection was performed using the enhanced chemiluminescence (ECL) kit (Amersham, Arlington Heights, IL, USA). Membranes were stripped using PBS + 0.1% Tween 20 + 0.1% SDS and rehybridized with rabbit anti-human ERK1/2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as a loading control. Western blotting experiments were repeated three times.

Electrophoretic mobility shift assay (EMSA)

For electrophoretic mobility shift assays, stimulated cells were harvested after 24 hours of culture. Nuclear extracts were prepared as previously described[27] and stored in aliquots at -80°C. Double-stranded synthetic oligonucleotide probes containing the Activator Protein 1 (AP-1) or Nuclear Factor -κB (NF-κB) consensus sequences (AP-1, 5'-AGC TGA AAT TCC AGA GAG

GAG-3'; NF-κB, 5'-AGC TGC GG GAT TTC CCT G-3') were ^{32}P-γATP (Amersham-Pharmacia Biotech) end-labelled with T4 polynucleotide kinase and used in the gel retardation assay. The AP-1 and NF-κB binding consensus sequences are underlined. For supershifts, 1 μg of rabbit polyclonal antibodies specific for NF-κB p50-subunit and p65-subunit were added (Santa Cruz Biotechnology). For AP-1 supershifts, antibodies specific for b-fos-subunit and pooled antibodies against c-jun and b-jun subunits (Santa Cruz Biotechnology) were added to the nuclear extracts. Electrophoretic mobility shift assays were performed as described using 10 μg of nuclear extract preparations.[27] Samples were loaded on 4.5% polyacrylamide gels and run for 1.5 h at 150 Volts in 0.5 x Tris-Borate-EDTA (TBE) buffer at room temperature. Gels were dried and exposed to Kodak XAR films (Eastman Kodak, Rochester, NY, USA) at -80°C with intensifying screen.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from 3 x 10^6 PCL cells after 96 hours of stimulation using Trizol™ reagent (Life Technologies) according to the manufacturers instructions. Approximately 1 μg of RNA was primed with 165 ng random hexamers (Amersham-Pharmacia Biotech) and Murine Moloney Leukaemia Virus reverse transcriptase (SuperscriptII™ Life Technologies) in a volume of 20 μl, containing 50 mmol/l KCl, 20 mmol/l Tris-HCl (pH = 8.4), 2.5 mmol/l $MgCl_2$, 1 mmol/l dithiothreitol (DTT) and 0.5 mmol/l of each deoxynucleotide triphosphate (dNTPs), for 50 min at 42°C. The RT reaction was terminated by enzyme inactivation for 5 minutes at 95°C. Integrity of cDNA was confirmed by β-Actin specific RT-PCR (β-Actin forward: 5'-TCA CCC ACA CTGTGC CCA TCT ACG A-3'; β-Actin backward: 5'-CAG CGG AAC CGC TCA TTG CCA ATG G-3'). In a 25 μl PCR reaction, 0.5 μl cDNA was amplified, containing 50 mmol/l KCl, 20 mmol/l Tris-HCl (pH = 8.4), 1.5 mmol/l $MgCl_2$, with 15 pmol of both primers, and 1.25 U Taq DNA polymerase (Life Technologies). IL-6 RT-PCR was performed at 55°C annealing temperature for 30 cycles. (IL-6 forward: 5'-TCA ATG AGG AGA CTT GCC TG-3'; IL-6 backward: 5'-GAT GAG TTG TCA TGT CCT GC-3'). PCR products were separated on LE agarose gels (Seakem, FMC Bioproducts, Rockland, MN, USA) and visualized by ethidium bromide staining and ultra violet illumination.

Statistical analysis

The Mann-Whitney *U*-test was used to determine significance of differences between groups. The student *t*-test for paired samples was used to determine significance of relative protein level data in Western blot experiments (P values < 0.05 were considered significant).

RESULTS

CD27-triggering on PCL cells is not involved in proliferation but inhibits spontaneous and dexamethasone-induced apoptosis.

To determine spontaneous and dexamethasone-induced apoptosis of CD27-stimulated PCL cells, PCL cells were cultured and subsequently analyzed by flow cytometry for annexin-V binding and intracellular active (cleaved) caspase 3. A significant lower percentage of apoptotic PCL cells was observed in the presence of the CD70-transfected cell line compared to the parental cell line, as assessed by the Annexin-V binding assay (mean 10.2 ± 1.1% *vs* 13.6 ± 1.1% early apoptotic PCL cells, $p < 0.05$) (Fig 1A), and the active caspase 3 assay (mean 4.5 ± 1.1% *vs* 9.9 ± 0.9%, $P < 0.05$) (Fig 1B). The anti-apoptotic effect of CD27-triggering was more evident when cells were treated with dexamethasone. CD27-triggering significantly inhibited dexamethasone (0.1 μM) induced apoptosis as determined by annexin-V binding (mean 14.1 ± 0.8% *vs* 21.0 ± 0.5%, $P < 0.05$) (Fig 1A) and active caspase 3 staining (mean 8.2 ± 0.8% *vs* 12.7 ± 0.9%, $P < 0.05$) (Fig 1b). These results demonstrate that the CD27/CD70 interaction can inhibit caspase 3 dependent apoptosis in PCL cells. We also examined the effect of CD27-triggering on proliferation by the CFSE-fluorescence decay assay. Purified CD27-expressing PCL cells were stimulated with the CD70-transfected cell line CD70/300-19.

After 72 and 120 hours of stimulation no decrease in CFSE fluorescence was observed (data not shown), showing that CD27-triggering on PCL cells did not modulate proliferation.

Anti-apoptotic properties of CD27/CD70 interactions are not mediated by the induction of autocrine IL-6 production.

IL-6 has been shown to be a paracrine and/or autocrine growth- and survival-factor for malignant plasma cells.[28-30] Malignant plasma cells are protected against dexamethasone- and FAS-induced apoptosis by IL-6.[31-33] To test whether the anti-apoptotic effect of CD27-triggering was linked to induction of autocrine IL-6 production, PCL cells were cultured with CD70/300-19 or wild/300-19 in the presence of neutralizing anti-IL-6 antibodies. No significant increase in the percentage of apoptotic plasma cells was observed in the presence of anti-IL-6 antibodies. Additionally, in response to CD27-triggering no induction of IL-6 mRNA expression was detected after 96 hours of culture, as assessed by IL-6 specific RT-PCR (Fig 2).

p38 MAPK and ERK1/2 are involved in CD27-mediated signal transduction in PCL cells.

Previous studies have shown that CD27-signalling is mediated by members of the TRAF family, finally resulting in the activation of JNK and NF-κB.[24,34] Furthermore, apoptosis of malignant plasma cells is associated with the activity of MAPK.[35,36] To investigate the involvement of p38 MAPK and ERK1/2 in CD27-mediated signalling, PCL cells were stimulated with CD70/300-19 or wild/300-19. By Western blotting using anti-phospho-p38 and anti-phospho ERK1/2 antibodies it was demonstrated that upon CD27-triggering, p38 was rapidly phosphorylated (within 5

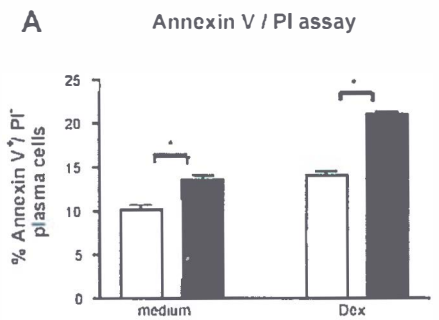
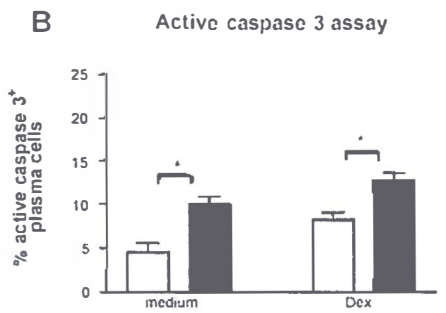


Figure 1. Triggering of CD27 on plasma cell leukaemia cell inhibits spontaneous and dexamethasone induced apoptosis. PCL cells were stimulated for 24 h with CD70/300-19 (open bars) or the control cell line wild/300-19 (black bars), with or without dexamethasone (0.1 μmol/l). Apoptosis was determined by annexin-V/propidium iodide staining (A) and staining for intracellular active caspase-3 (B). Experiments were performed three times, representative experiments are shown. Results are given as means ± standard deviations of percentage apoptotic cells (* $P < 0.05$).



min). Furthermore, CD27-induced p38 activation coincided with downregulation of ERK1/2 phosphorylation, which may suggest that activated p38 was involved in the inhibition of ERK1/2 phosphorylation. A similar cross talk between p38 and ERK1/2 has been described earlier.[37,38] In dexamethasone treated PCL cells a sustained activation of p38 was observed irrespective of CD27 stimulation. The results of three independent experiments are depicted in Figure 3.

To further delineate the involvement of p38 MAPK and ERK1/2 in CD27-mediated signalling, PCL cells were pre-incubated with specific inhibitors of p38 phosphorylation (SB203580) and ERK1/2 phosphorylation (U0126) and subsequently stimulated with CD70/300-19 or wild/300-19 in the presence of dexamethasone. Flow-cytometric analysis for active (cleaved) caspase 3 showed that CD27-triggering resulted in a 50% inhibition of apoptosis, this inhibition was decreased to 25% in SB203580 or U0126 treated cells. In cultures in which both inhibitors were present, CD27-triggering did not inhibit dexamethasone-induced apoptosis (Fig 4A). Addition of inhibitors had no toxic effects as assessed by apoptosis assays performed on wild/300-19 cultures. As previously described, Western blotting analysis was carried out to show that the used concentrations effectively blocked the phosphorylation of p38 and ERK1/2.[39-42] These data suggests that p38 and ERK1/2 were both associated with CD27-mediated signalling and were involved in the anti-apoptotic properties of CD27-triggering on PCL cells.

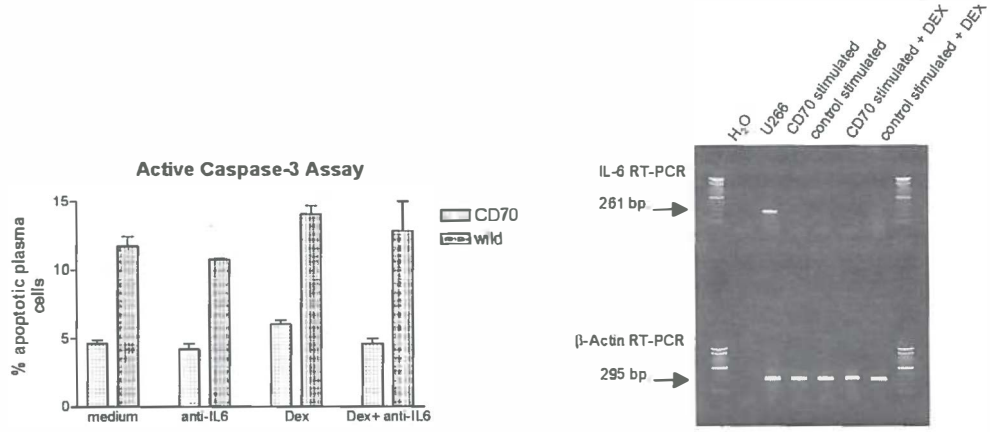


Figure 2. Anti-apoptotic effects of CD27 triggering on plasma cell leukaemia cells are not mediated by induction of interleukin 6 expression. (A) PCL cells were stimulated for 24 h with CD70/300-19 or the control cell line wild/330-19, with or without dexamethasone and neutralizing anti-IL-6 serum. (B) IL-6 specific RT-PCR on stimulated PCL cells. The human myeloma cell line U266 was used as a positive control. β-Actin RT-PCR was performed to confirm cDNA integrity.

Inhibition of p38 MAPK and ERK1/2 phosphorylation sensitizes PCL cells for dexamethasone-induced apoptosis.

Inhibition of p38 and ERK1/2 phosphorylation had a strong sensitizing effect on dexamethasone-induced apoptosis of PCL cells. Dexamethasone-treatment induced a two-fold increase of apoptotic PCL cells, whereas in PCL cells pre-treated with SB203580 and U0126, dexamethasone induced a 10-fold increase of apoptosis compared with untreated PCL cells and a fivefold increase compared to PCL treated with dexamethasone alone (Fig 4B). This effect was less pronounced when each of the inhibitors was added alone (2.5-fold). This suggests that p38 and ERK1/2 both mediate the anti-apoptotic stress-response following dexamethasone-treatment in PCL cells.

CD27-triggering on PCL cells is associated with persistent DNA-binding of Activator Protein 1 (AP-1) but not Nuclear Factor-κB (NF-κB).

Earlier studies have shown that activation of p38 MAPK is often associated with activation of the stress activated protein kinase (SAPK/JNK). Furthermore, overexpression and ligation of CD27 could activate SAPK/JNK resulting in the phosphorylation of c-jun and the subsequent activation of AP-1 and NF-κB.[24] We questioned whether DNA binding activity of AP-1 and/or NFκ-B is modulated by CD27-triggering on PCL cells. PCL cells were cultured for 24 hours with CD70/330-19 or wild/300-19. A constitutive nuclear presence of the AP-1 complex and the NF-κB complex was detected in the absence of dexamethasone. CD27-triggering had no apparent effect on the constitutive DNA binding of AP-1 and NF-κB. However, when the PCL were

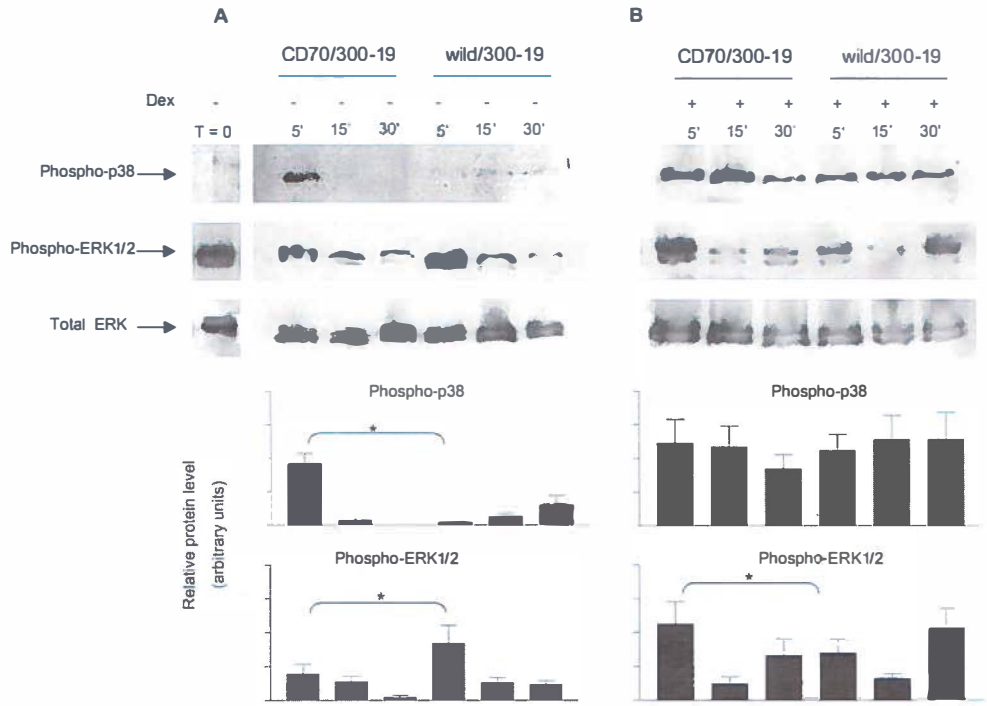


Figure 3. Effects of CD27 triggering and dexamethasone treatment on p38 and ERK1/2-phosphorylation in PCL cells. PCL cells were stimulated with CD70/300-19 or wild/300-19 without (A) or with (B) 0.1 μM dexamethasone for the indicated periods. Immunoblotting was performed with anti-phospho- p38, anti-phospho ERK1/2 and anti-total ERK1/2. Relative protein levels were measured by densitometry and normalized for the amount of total ERK1/2 signals. Experiments were performed in triplicate; graphs depict means ± standard deviations. (* $p < 0.05$).

treated with dexamethasone, AP-1 was downmodulated in the wild/300-19 cultures, whereas it was unaltered in the CD70/300-19 cultures (Fig 5A). These findings indicate that CD27-triggering inhibited dexamethasone-induced downmodulation of AP-1. In contrast, CD27-triggering had no effect on the constitutive NF-κB DNA binding. In concordance with previous reports dexamethasone treatment downmodulated NF-κB DNA binding.[43] CD27-triggering was not able to prevent this downmodulation in PCL cells. Supershift experiments with antibodies against NF-κB p50 and p65 specific subunits revealed no significant differences in CD27-triggered and control-stimulated PCL cells. The NF-κB complex predominantly consisted of p50/p50 homodimers and p50/p65 heterodimers (Fig 5B). These results suggest that inhibition of dexamethasone-induced AP-1 downmodulation might be involved in the anti-apoptotic properties of CD27-triggering in PCL cells.

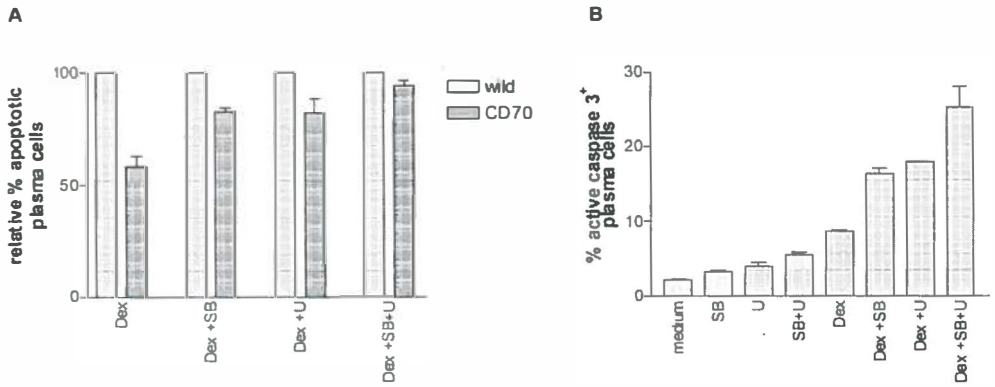


Figure 4. Specific blockade of p38 and ERK1/2 activation abrogates CD27-mediated antiapoptotic effect and sensitizes PCL cells to dexamethasone-induced apoptosis. PCL cells were stimulated for 24 h with CD70/300-19 or wild/300-19 and 0.1 μmol/l dexamethasone. The p38 MAPK competitive inhibitor SB203580 was added at a final concentration of 1.0 μmol/l. The ERK1/2 inhibitor U0126 was added at a final concentration of 2.0 μmol/l. (A) Relative percentage of apoptotic PCL cells is depicted, apoptosis of PCL cells stimulated with wild/300-19 was set to 100%. (B) PCL cells were stimulated for 24 h with CD70/300-19 or wild/300-19 with or without dexamethasone and SB203580 and U0126. Absolute percentages of active caspase-3 positive PCL cells are shown. Experiments were performed in triplicate; results are given as means ± SD. Apoptosis of PCL cells was determined by three-colour FACS staining for CD138, CD38 and intracellular caspase-3.

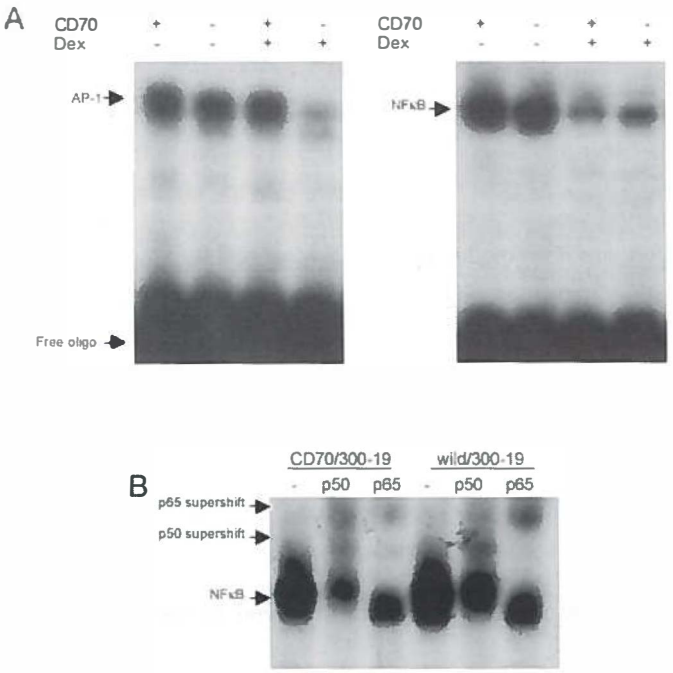


Figure 5. Electrophoretic mobility shift assays for AP-1 and NF–κB binding in plasma cell leukaemia cells. Effects of CD27 triggering and dexamethasone treatment. (A) Nuclear extracts of stimulated cells were subjected to electrophoretic mobility shift assays with AP-1 and NF-κB specific oligonucleotides. CD70 stimulation of PCL cells inhibited dexamethasone induced downregulation of AP-1 but not of NF-κB. The same nuclear extract preparations were used in both experiments.
(B) Supershift experiments using NF-κB p50 and p65 specific antibodies. PCL cells were stimulated with CD70/300-19 or the control cell line wild/300-19.

DISCUSSION

Several reports suggest that primary PCL can be considered as a separate disease entity that has several biological and clinical characteristics, which are different from MM.[4-7] In a previous study we demonstrated that CD27 is highly expressed in primary PCL, whereas its expression is generally lost in relapsed and progressive MM.[11] The functional role of CD27 expressed on malignant plasma cells however remained unclear. Therefore, we set out to analyse the functional consequences of CD27-triggering on PCL cells. The data presented in this report show that triggering of CD27 on PCL cells by its natural ligand CD70 has anti-apoptotic effects, most prominently in the presence of dexamethasone. This anti-apoptotic effect might be involved in the clonal selection of CD27-expressing PCL cells, which is obviously not operable in MM. CD27 is expressed on many cell types[15,44] and the CD27/CD70 interaction is implicated to play a role in enhancing as well as in inhibiting apoptosis. For instance, CD27/CD70 interaction counteracted the apoptotic effects of IL-10 in memory B cells, thereby enabling end-stage differentiation of these cells.[20] Concurrently, ligation of CD27 may also result in the recruitment of the pro-apoptotic molecule Siva that may lead to an apoptotic effect.[45]

Next, we questioned which downstream signal transduction pathways were involved in the anti-apoptotic properties of CD27-triggering in PCL cells. Akiba *et al.*[24] demonstrated that CD27 overexpression results in the activation of NF-κB and JNK. Gravestein *et al.*[34] showed by yeast-two-hybrid screenings that the cytoplasmic tail of CD27 communicated with JNK via TRAF2. In MM, the activation of JNK is linked to apoptosis. Inhibition of the JNK pathway protected MM cells from FAS induced apoptosis whereas it had a sensitizing effect on dexamethasone induced apoptosis.[36] Furthermore, the activation of p38 MAPK was associated with IL-6 mediated signalling enhancing MM cell growth and survival.[47]

In this report we show that CD27-triggering on PCL cells resulted in a rapid activation of p38 which negatively regulates the activation of ERK1/2. In addition, specific inhibition of p38 by SB203580 resulted in a marked upregulation of ERK1/2 phosphorylation suggesting cross talk between these MAP kinases, as previously demonstrated in monocytic cells and in neutrophils.[27,46] The phosphorylated p38 α-isoform can interact directly with ERK1/2 and inhibits its phosphorylation by steric hindrance.[37,38]

Dexamethasone upregulated p38 phosphorylation in both CD27-triggered as well as in control stimulated PCL cells suggesting that p38 was involved in the stress response following dexamethasone treatment. CD27-triggering of dexamethasone treated cells shortly abolished the negative regulatory effect of p38 on ERK1/2 phosphorylation, resulting in an increase of ERK1/2 activation. Whether this short modulation is instrumental in the cellular effects of CD27-triggering in dexamethasone treated cells remains to be elucidated. It is possible that dexamethasone treatment and CD27-mediated signalling target different p38 isoforms that have differential effects on ERK1/2 activation. In parallel, it has been shown in neutrophils that inhibition of p38 increased the anti-apoptotic effect of lipopolysaccharide, which was

mediated through the enhancing effect on ERK1/2 activation.[46]

At the cellular level, specific inhibition of p38 and ERK1/2 activation by SB203580 and U0126, significantly diminished the anti-apoptotic effect of CD27-triggering, confirming that the activation of MAPK plays a pivotal role in the CD27-mediated signalling and its cellular consequences in PCL cells. Furthermore, simultaneous inhibition of p38 and ERK1/2 strongly sensitized PCL cells for dexamethasone-induced apoptosis. It has been demonstrated that the proteasome inhibitor PS-341, inhibits ERK1/2 activation, in addition to the inhibitory effect on IκBα degradation, resulting in sensitization of MM cells for dexamethasone-induced cell death.[48] Additionally, in a recent study it was shown that targeting of p38 MAPK by the specific inhibitor VX-745 inhibited MM cell growth by decreasing paracrine IL-6 secretion in bone marrow stromal cells.[49] However, PCL cells grow independently of the bone marrow microenvironment. This study shows that endogenous MAPK can also be regarded as an attractive target to overcome drug resistance in primary PCL.

Given that IL-6 is the most important growth and survival factor in MM, we examined whether IL-6 is involved in the anti-apoptotic effect of CD27-triggering on PCL cells. Neutralizing anti-IL-6 antibodies did not affect the anti-apoptotic effect of CD27-triggering. Additionally, RT-PCR analysis revealed that the anti-apoptotic members of the Bcl-2 protein family, Bcl(X_L) and Mcl-1, were not involved in the anti-apoptotic effect of CD27-triggering (data not shown). We hypothesize that the activation of the Raf/MEK/MAPK signalling cascade is (partly) mediated through the ligation of CD27, in an IL-6 independent manner.

Previous studies have shown that NF-κB and AP-1 are important downstream targets of the MAPK signal tranduction pathway.[27,50-53] These transcription factors have been implicated in the survival and proliferation of malignant plasma cells.[43,54] In this study, DNA binding activity of NF-κB and AP-1 was downregulated by dexamethasone treatment, in concordance with earlier studies.[43,55,56] CD27-triggering inhibited the dexamethasone-induced downregulation of AP-1 without an effect on NF-κB. Our data demonstrated that CD27-triggering modulated AP-1 DNA binding activity via the MAPK signalling pathway and that persistent AP-1 DNA binding activity was associated with protection from dexamethasone-induced apoptosis.

In summary, this study identified the functional role of CD27 expression on primary PCL cells, in which CD27-triggering protected PCL cells from dexamethasone-induced apoptosis. The loss of CD27 expression from relapsed and progressive MM cells indicates that CD27 expression is differentially regulated in MM and primary PCL, suggesting that primary PCL is separate disease entity. We demonstrated that the MAPK signal transduction pathway is involved in CD27-triggering in PCL cells and showed that specific inhibition of this pathway results in sensitization for dexamethasone, prompting to explore the possibilities of targeting this pathway in order to overcome drug resistance of malignant plasma cells.

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chapter eight

General discussion and future perspectives

In this thesis the clinical significance and functional aspects of clonotypic B cells and clonotypic Ig-isotype variants in multiple myeloma patients were studied. For decades, the field of myeloma biology and clinical research has endeavoured to understand the problem of the myeloma clone persistence and resistance, and its clinical implications. However, despite these efforts, treatment options for this disease are limited and a curative treatment is as yet not frequently available. The discovery of circulating B cells expressing identical Ig receptors as the malignant plasma cells in MM patients gave rise to the thought that in MM a precursor population is present. According to this hypothesis, the clonotypic precursor population reflects a chemoresistant and proliferative reservoir of disease, responsible for relapse observed in almost all patients. Sensitive molecular biology techniques demonstrated that clonotypic B cells expressed identical Ig rearrangements, providing indisputable evidence for the clonal relationship between these circulating B cells and the myeloma plasma cells in the bone marrow. Of great importance was the notion that the myeloma clone had acquired V(D)J somatic hypermutations without further intraclonal diversification, pointing towards a post-germinal center origin.[1,2] The stability of the clonal Ig rearrangement throughout the course of the disease substantiated the lack of clonal evolution within the myeloma clone.[3] The discovery of clonotypic cells expressing different Ig-isotypes indicates that the myeloma cell of origin has past the stage at which somatic hypermutations are acquired, but might still have the capacity to undergo immunoglobulin class switch recombination.[4-6] However, direct clinical evidence that circulating clonotypic B cells are instrumental in the pathogenesis of MM is still lacking. It has been reported that the majority of the peripheral blood B cells in MM patients express clonotypic V(D)J rearrangements.[7,8] However, these results could not be reproduced by other research groups.[9-11] To date, the most convincing data suggest that clonotypic B cells constitute only a minority of the peripheral blood B cells.[9] This can be brought into conformity by the hypothesis that clonotypic B cells continuously undergo (directed) differentiation towards plasma cells, thereby replenishing the tumour population. Alternatively, clonotypic B cells could be considered as the non-malignant circulating clonal by-products of the germinal center reaction that gave rise to the myeloma clone. The key question that arises however, is to what extend myeloma clonotypic B cells share malignant features with the myeloma plasma cells. The low number of clonotypic B cells in MM patients, and the fact that they can only be distinguished from normal B cells on basis of the expressed Ig-idiotype, complicates the isolation and in-depth analysis of these cells. We aimed to determine the involvement of clonotypic B cells expressing variant Ig-isotypes in relapse of the disease. Studying 37 MM patients undergoing autologous stem cell transplantation after high-dose combination chemotherapy, a high incidence of oligoclonal serum Igs was demonstrated **[chapter 3]**. In 48% of the cases the oligoclonal serum Igs were transient and disappeared after a median follow-up of 22 months. In the remaining 52% they persisted with a median follow-up of 31 months. Follow-up showed that there was no significant difference in the overall and event-

free survival between the subgroups. The reappearance (and persistence) of monoclonal serum Ig after treatment is an important sign of relapse and has implications for further treatment decisions. However, the clinical relevance of oligoclonal serum Igs after treatment was unknown. They could be the result of expansion and differentiation of clonotypic Ig-isotype variants, and therefore, considered as a marker of relapse of the disease. To test this hypothesis we obtained bone marrow before and after autologous stem cell transplantation from seven MM patients with persistent oligoclonal serum Igs and assessed the presence of clonotypic Ig-isotype variants by sensitive patient- and isotype-specific RT-PCR **[chapter 4]**. In all seven cases we detected the original 'clinical' clonotypic Ig-isotype in the pre-transplantation bone marrow. In two IgG-expressing MM cases additional clonotypic Ig-isotype variants were detected. In one of these cases an IgA-expressing clonotypic variant was found whereas in the other case IgM- and IgA-expressing clonotypic variants were detected. In post-transplantation bone marrow samples the original clonotypic Ig-isotype was detected in 6/7 cases. Clonotypic Ig-isotype variants were not found in post-transplantation bone marrow, nor in peripheral blood stem cell samples collected for autografting these patients. These findings argue against the clonal outgrowth of clonotypic Ig-isotypes post-treatment. We conclude that the appearance of oligoclonal serum Igs is not related to relapse of the disease but rather reflects the dysclonal recovery of normal B cells in patients that underwent high-dose chemotherapy followed by autologous stem cell reinfusion. Recently, in a large clinical study, it was shown that after high-dose chemotherapy and autologous stem cell reinfusion the presence of abnormal protein bands upon serum immunofixation electrophoresis is a favourable feature, imposing a better prognosis for MM patients.[12] This supports our findings, and underscores that serum oligoclonality in MM patients is related to recovery of Ig production and not Ig-isotype switching within the myeloma clone or outgrowth of clonotypic Ig-isotype variants. To further characterize functional aspects of clonotypic B cells (expressing variant Ig-isotypes) we studied their *in vitro* proliferative capacity and differentiation properties **[chapter 5]**. We therefore employed the CD40 culture system, capable of sustaining long-term B-cell growth *in vitro*,[13,14] to culture peripheral blood B cells from untreated MM patients. B cells were cultured for 7 and 14 days in the presence of irradiated CD40L-transfected mouse fibroblasts and IL-4 to induce B-cell proliferation. The presence of clonotypic B cells expressing variant Ig-isotypes before and after culturing was determined by patient- and Ig-isotype specific RT-PCR. In 3 out of 6 studied patients, clonotypic Ig-isotype variants were found in the peripheral blood. Only in these cases, the same clonotypic Ig-isotype variants were detected after culturing, suggesting that they survived and proliferated in the CD40 culture system but probably not outgrow normal B cells. Furthermore, a clonotypic B-cell subclone could be isolated by limiting dilution *in vitro*; this subclone expressed the original Ig-isotype. However, the success-rate of obtaining such a clone was approximately 1%, indicative for the low frequency of clonotypic B

cells in the peripheral blood. These findings demonstrate that the variant Ig-isotypes are expressed at the circulating B-cell stage, which in accordance with the presence of V(D)J somatic hypermutations, suggesting a memory B-cell origin. To characterize the capacity of clonotypic B cells to differentiate into functional end-differentiated Ig-secreting cells, CD40-ligation was interrupted and IL-2 and IL-10 were added after 7 days of culture. The numbers of clonotypic B cells were determined by quantitative allele-specific oligonucleotide (ASO) PCR after 7 days of CD40L and IL-4 stimulation, and after the subsequent 7 days of stimulation with IL-2 and IL-10. Functional end-differentiation of cultured cells was shown by phenotypic maturation (CD20 downmodulation and CD38 upregulation), and induction of Ig-secretion. Studying the peripheral blood B cells from two patients it was shown that the relative frequency of clonotypic B cells was unaltered after CD40L and IL-4 stimulation. This finding demonstrated that the proliferative response of clonotypic B cells was equivalent to the normal non-clonotypic B cells. This was in contrast with the results obtained with mantle cell lymphoma cells, showing an enhanced proliferative response to CD40-ligation.[15-17] After induction of end-differentiation a significant decrease in the relative number of clonotypic B cells was observed. The inability of clonotypic B cells to undergo *in vitro* end-differentiation might be caused by the nature of differentiation stimuli that were provided in these experiments, resulting in preferential survival and differentiation of normal non-clonotypic B cells. This would suggest that clonotypic B cells differ in their requirements to achieve end-differentiation. Alternatively, it may imply that myeloma clonotypic B cells have lost the ability to differentiate as a consequence of 'arrested development', which is an important characteristic of many (B-cell) malignancies. For one patient it was demonstrated by fusion-product RT-PCR that the t(4;14) translocation, which was proven to be present in the bone marrow myeloma plasma cells, was also present in purified peripheral blood B cells. The translocation remained detectable after CD40L and IL-4 stimulation and after subsequent induction of end-differentiation. However, the fact that clonotypic B cells share chromosomal abnormalities with the malignant plasma cells in the bone marrow does not necessarily imply that they can be considered malignant. Based on the *in vitro* experiments, it remains questionable whether clonotypic B cells replenish the malignant plasma cell pool in the bone marrow. Apparently, clonotypic B cells clearly differed regarding the differentiation capacity in comparison with normal B cells, which argues against the hypothesis that these cells are myeloma-precursor cells that undergo continuous differentiation. The memory B-cell is the presumed cell of origin for the myeloma clone. Recently, CD27 has been identified as surface marker for human memory B cells.[18] To assess whether CD27 expression discerned clonotypic B cells from non-clonotypic B cells, $CD27^+$ (memory) and $CD27^-$ (naive) peripheral blood B cells were purified from two untreated MM patients. Genomic DNA was isolated and subjected to quantitative ASO-PCR. In both patients the relative frequency of clonotypic B cells was comparably low (<1%) in $CD27^+$, as well as in $CD27^-$ purified B cell populations (unpublished results), implying that clonotypic B cells can be $CD27^+$ as well as

CD27$^-$. These results suggest that B cells in MM may acquire somatic hypermutations without acquisition of surface CD27 expression. Alternatively, clonotypic B cells in MM might have lost expression of CD27. To gain more insight into the role of CD27 expression in MM patients, bone marrow samples from 28 MM patients were analysed by four-colour flow cytometry **[chapter 6]**. We demonstrated that CD27 was expressed heterogeneously on myeloma plasma cells. In patients in complete clinical remission a significant higher percentage of plasma cells expressed CD27 compared with patients at diagnosis, relapse or in partial remission. Additionally, cDNA microarray experiments demonstrated that CD27 mRNA was heterogeneously expressed in purified bone marrow plasma cells obtained from 74 newly diagnosed MM patients. In hierarchical clustering defined MM patient risk-groups, CD27 mRNA was shown to be expressed lowest in the high-risk MM patient-group, and highest in the low-risk group. These data suggest that loss of CD27 expression from MM plasma cells imposes a poor prognosis. By flow cytometry we demonstrated that bone marrow plasma cells from healthy donors and MGUS patients were characterized by high CD27 expression. None of the 9 human MM cell lines (which are derived from patients suffering from end-stage progressed MM) expressed CD27 at the protein and mRNA level. These findings were also confirmed by cDNA microarray, showing that CD27 was the second most downregulated gene in MM plasma cells compared with healthy donor plasma cells. Consequently, the high percentage of CD27 expressing plasma cells in patients that achieved complete clinical remission may be indicative for the reappearance of normal bone marrow plasma cells. In the majority of newly diagnosed MM patients distinct CD27$^+$ and CD27$^-$ plasma cell populations could be distinguished. Both populations were highly Ig-light chain restricted, suggesting that both belonged to the myeloma clone. This was confirmed by ASO-PCR on flow-sorted CD27$^+$ and CD27$^-$ plasma cells. Apparently, next to CD27$^-$ also CD27$^+$ clonal plasma cells are present in MM patients. We demonstrated that CD27 expression correlated

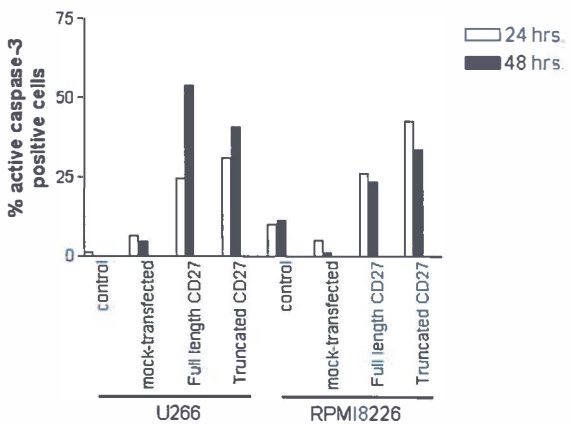

Figure 1. Transfection of full length or truncated CD27 leads to apoptosis of human MM cell lines. U266 and RPMI8226 were transfected with 1μg of pcDNA3.1 (mock-transfected), pcDNA-FLCD27 (full length CD27) or pcDNA-TRCD27 (truncated CD27). Apoptosis of transfected myeloma cells was determined by intracellular staining for active (cleaved) caspase-3.

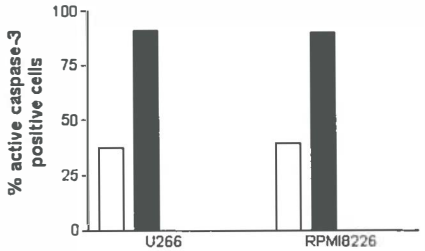

Figure 2. Induction of human MM cell line apoptosis by human Burkitt's lymphoma cell line. CD70-expressing human MM cell lines U266 and RPMI8226 were co-cultured with CD27-expressing Burkitt's lymphoma cell line Raji. Apoptosis of plasma cells was determined by assessing intracellular staining for active (cleaved) caspase-3 within the $CD138^{+}$ $CD38^{++}$ plasma cell gate.

significantly with CD19 expression on plasma cells, suggesting that loss of CD27 is specific for $CD19^{-}$ myeloma plasma cells. Additionally, this suggests that CD27 is expressed by the more immature plasma cells in MM. It is unknown whether $CD27^{-}$ plasma cells are derived from $CD27^{+}$ plasma cells or whether they represent separate self-replenishing populations. The presence of $CD27^{+}$ as well as $CD27^{-}$ clonotypic B cells in the peripheral blood may suggest that $CD27^{+}$ and $CD27^{-}$ myeloma plasma cells are derived from separate precursor populations. We speculate that during progression of the disease, $CD27^{-}$ plasma cells outgrow $CD27^{+}$ plasma cells for yet unknown reasons. Our results suggest that CD27 expression is an unfavourable characteristic for MM plasma cells. In this respect, loss of CD27 is the subject of clonal selection during progression. To address the question why CD27 expression is lost from myeloma plasma cells, we transfected the human MM cell lines U266 and RPMI8226 with the full length human CD27 cDNA and with truncated CD27, lacking the cytoplasmic tail necessary for CD27-mediated signal transduction. Enforced expression of either full length of truncated CD27 induced apoptosis in MM cell lines as determined by caspase-3 cleavage (5-6 fold increase compared with mock-transfected cells; Figure 1; unpublished results). This apoptotic effect was evidently not due to CD27-mediated signal transduction. Both U266 and RPMI8226 strongly expressed CD70, the natural ligand for CD27. We speculated that the apoptotic effect of CD27-transfection in these cell lines was the result of CD70-mediated signal transduction. To test this hypothesis, we co-cultured both cell lines with the Burkitt's lymphoma cell line Raji, which is highly positive for CD27, and was used as a source for membrane-bound CD27. Upon co-culture, massive apoptosis of the MM cell lines was observed, providing additional evidence that the CD70-CD27 interaction was involved in the induction of plasma cell apoptosis (Figure 2; unpublished results). These data suggest that CD27-mediated crosslinking of CD70 is involved in myeloma plasma cell apoptosis, providing a rationale for the downmodulation of either CD27 or CD70. Preliminary flow cytometry results show that myeloma plasma cells express low levels of CD70, without apparent heterogeneity in our cross-sectional patient group (unpublised results). Of interest is the fact that some human myeloma cell lines highly expressed CD70 whereas others showed low expression levels[19] (own unpublished observations). Gene expression

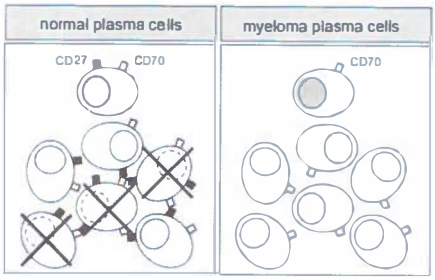

Figure 3. Proposed model for CD27-CD70 mediated plasma cell regulation. Normal plasma cells co-express CD27 and CD70, facilitating homo- and heterotypic interactions involved in plasma cell apoptosis. Plasma cells stimulated through the CD70 receptor undergo apoptosis. Due to loss of CD27 expression myeloma plasma are insensitive to CD70-mediated apoptosis.

profiling experiments comparing healthy donor mature plasma cells with *in vitro* generated polyclonal plasmablastic cells demonstrated that CD70 was downregulated in mature plasma cells. Polyclonal plasmablastic cells co-expressed CD27 and CD70, suggesting that an autocrine/paracrine differentiation loop in these cells might exist. We speculate that the CD27-CD70 interaction might be important in regulating the size of plasma cell clones in the normal bone marrow, by facilitating homotypic and/or heterotypic interactions (Figure 3). It is conceivable that disruption of this homeostatic mechanism is involved in the development of plasma cell malignancy. Recently, it has been shown in a transgenic mouse model that B cell-specific CD70-transgene over-expression leads to a rapid depletion of B cells. Additional experiments demonstrated that this depletion is CD27-CD70 dependent. It was shown that CD70-transgenic B cells activated IFNγ-producing T cells in a CD27-dependent manner.[20] We hypothesize that besides the immune-mediated cell death, the CD70-CD27 interaction also has intrinsic apoptotic properties, which may play an important role in plasma cell homeostasis.

The low expression or absence of CD27 on myeloma plasma cells in progressive disease is not in line with the results we obtained in *de novo* plasma cell leukaemia (PCL). Studying three cases, we found homogeneous high expression of CD27 on PCL plasma cells, in spite of the aggressive disease characteristics and dismal outcome.

To determine the functional role of CD27 expression, PCL cells were stimulated *in vitro* with CD70. **[chapter 7]**. It was shown that CD27-triggering had anti-apoptotic effects that involved the p38 and ERK1/2 mitogen activated protein kinases (MAPK). Specific inhibition of p38 and ERK1/2 abolished the anti-apoptotic effect and resulted in sensitization for dexamethasone-induced apoptosis. CD27-triggering on dexamethasone-treated PCL cells resulted in persistent DNA-binding activity of activator protein 1 (AP-1) without affecting downmodulation of nuclear factor-κB (NF-κB). Previous studies demonstrated that *de novo* PCL differed from MM regarding immunophenotype and genetic alterations,[21-25] suggesting that it may represent a separate disease entity. It remains to be established whether expression of CD27 can regarded as a specific marker for *de novo* PCL. Our results clearly indicate that CD27 expression is regulated differently in *de novo* PCL compared with MM. CD27-triggering on PCL cells had anti-apoptotic

effects, providing a rationale for retainment of CD27 expression on PCL cells. Alternatively, it is conceivable that unlike MM plasma cells with predominant focal growth in the bone marrow, PCL cells in the peripheral blood undergo homotypic interactions to a lesser extend. Therefore, PCL cells may be less susceptible to homotypic CD70-mediated apoptosis, which may clarify the lack of CD27 downregulation. The anti-apoptotic effects of CD27-triggering were not mediated by the induction of IL-6 production. However, known downstream effectors of IL-6 signalling in MM were activated upon CD27-triggering. Sensitization of dexamethasone-induced apoptosis by specific inhibition of p38 and ERK1/2 suggest that these MAPK are interesting novel therapeutic targets to overcome drug resistance in PCL, as has been demonstrated for MM.[26,27]

FUTURE PERSPECTIVES

To date, the indications that clonotypic B cells are indispensable for myeloma development, persistence and progression remain disputable. The studies presented in this thesis question the role of clonotypic B cells (expressing variant Ig isotypes) in the pathogenesis of MM. Neither clonal expansion nor apparent plasma cell differentiation of clonotypic B cells have been reported to take place *in vivo* or *in vitro*. In a recently developed xenograft-model using immunodeficient mice, it has been demonstrated that mobilized blood from MM patients could give rise to MM-like symptoms, even when hematopoietic progenitors were enriched by $CD34^+$ selection.[28,29] However, from these experiments it cannot be concluded with certainty that circulating clonotypic B cells, and not residual contaminating plasma cells, were responsible for the MM related phenomena. It would be of interest to assess to what extend highly purified circulating B cells from the peripheral blood of MM patients have the ability to invoke MM-like symptoms in the xenograft mouse model. More stringent purging strategies to remove clonotypic B cells from mobilized stem cell autografts should be explored and preferentially be applied in combination with therapeutic strategies to accomplish complete B-cell eradication in MM patients. Anti-CD20 antibody (rituximab) treatment in combination with IFNγ is well-tolerated in MM patients, and offers an applicable treatment modality to target clonotypic B cells.[30] The Ig-idiotype can be regarded as the most specific target for clonotypic B cells. However, the development of anti-idiotype antibodies is cumbersome. Idiotype vaccination is more convenient and provides an attractive approach to target clonotypic B cells,[31] and thus needs to be explored in clinical settings.

In addition, the efficacy of other monoclonal antibodies to target myeloma plasma cells and presumed precursor cells should be tested. For instance, Campath-1H is a humanized monoclonal antibody that targets CD52 and is active in chronic lymphocytic leukaemia.[32,33] In a recent study it was reported that CD52 is also expressed in MM, especially on clonal immature $CD45^+$ plasma cells, providing a basis for the evaluation of the therapeutic benefits of Campath-1H in MM.[34] Our findings suggests that CD70 might also be an attractive target for immunotherapy.

Other studies have demonstrated that CD70 expression is an important co-stimulatory pathway involved in anti-tumour responses.[35,36] Co-stimulatory pathways and stimuli that result in upregulation of CD70 expression on MM cells need to be investigated. It has been shown that CD40-stimulation upregulated CD70 expression on MM plasma cells,[37] it remains to be established whether this upregulation results in an increased susceptibility to CD27-mediated apoptosis. In order to establish whether loss of CD27 expression in MM is of prognostic value in MM, careful assessment of CD27 expression on plasma cells in a controled clinical trial is required. Currently, we are determining CD27 expression on myeloma plasma cells in a clinical setting. Our finding that specific inhibition of p38 and ERK1/2 MAPK sensitizes PCL cells for dexamethasone induced apoptosis exemplifies that drug resistance can be overcome, at least partially. Pharmacological and immunological targetting of pathways involved in resistance and apoptosis in combination with conventional therapy is promising and should be explored further. Recently, encouraging results have been obtained with novel therapeutic agents disrupting specific signalling pathways involved in survival and growth of the myeloma clone. The recent application of microarray technologies has demonstrated the heterogeneity between MM patients and within the myeloma clone.[38-40] This technology allows the comprehensive identification of deregulated pathways and tumour-specific targets in MM, facilitating the development of novel drugs. Furthermore, in near future, the use of these technologies will become more widespread so that they can be employed to assess whether MM patients are eligible for newly developed treatment modalities. In addition, signalling pathways are often deregulated at the protein level. Signalling pathways consist of cascades of protein interactions and modifications like phosphorylation/dephosphorylation or conformational changes, which eventually affects downstream mRNA expression. The current developments in the field of proteomics expedites the assessment of protein alterations in a high-throughput fashion. These technical advances will further contribute to the elucidation of the disturbed pathways in MM, and hopefully will lead to the development or identification of new drugs targetting these pathways.

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Nederlandse samenvatting

Multipel myeloom (ook wel de ziekte van Kahler) is een kwaadaardige ziekte die wordt gekenmerkt door de aanwezigheid van kwaadaardige plasmacellen in het beenmerg. Ondanks het feit dat deze plasmacellen het dominante celtype is in multipel myeloom patiënten, die met name verantwoordelijk zijn voor de ziektesymptomen, is uit verschillende studies gebleken dat in myeloom patiënten ook minder uitgerijpte B cellen aanwezig zijn die tot dezelfde kloon behoren als de kwaadaardige plasmacellen. Het onderzoek beschreven in dit proefschrift heeft als doel de heterogeniteit aan betrokken celtypen te karakteriseren en te onderzoeken in welke hoedanigheid deze celtypen bijdragen aan het multipel myeloom ziekteproces.

Onder normale omstandigheden zijn plasmacellen verantwoordelijk voor de productie van antistoffen (immunoglobulinen) en spelen dientengevolge een belangrijke rol in het afweersysteem. Plasmacellen worden gevormd door uitrijping van B cellen. B cellen ontstaan in het beenmerg vanuit hematopoïetische stamcellen, waarna ze naar de bloedsomloop migreren. Wanneer B cellen in contact komen met lichaamsvreemde stoffen migreren ze naar de secundaire lymfoïde weefsels en kunnen daar onder invloed van omgevingsfactoren uitrijpen tot plasmacellen of geheugen B cellen. Deze plasmacellen en geheugen B cellen zijn in de lymfoïde weefsels geselecteerd op basis van hun vermogen lichaamsvreemde stoffen te herkennen. De geheugen B cellen vervolgen hun weg naar de bloedsomloop en zijn verantwoordelijk voor langdurige immuniteit. De plasmacellen migreren terug naar het beenmerg waar ze verder uitrijpen tot cellen die antistoffen uitscheiden. In het beenmerg van gezonde personen maken plasmacellen minder dan 1% uit van het geheel aan kernhoudende cellen. Bij multipel myeloom patiënten is dit percentage veel hoger ten gevolge van een klonale woekering van plasmacellen. De klonale woekering is verantwoordelijk voor een sterk verhoogde concentratie van één bepaalde antistof in het bloed (en soms in de urine) van myeloom patiënten. Dit wordt het paraproteïne genoemd. In de meeste myeloom patiënten produceren de kwaadaardige plasmacellen immunoglobulinen van het IgG isotype, in een minderheid van de patiënten is het paraproteïne van het IgA isotype. In sommige patiënten produceren de kwaadaardige plasmacellen alleen een lichte keten fragment van het immunoglobuline-molecuul, het zogenaamde Bence-Jones eiwit. Patiënten met paraproteïnen van het IgM, IgD of IgE isotype zijn zeer zeldzaam. Het paraproteïne is naast de aanwezigheid van een verhoogd aantal plasmacellen in het beenmerg een belangrijk kenmerk voor de diagnose en prognose van de ziekte. Het succes van een behandeling kan gedeeltelijk worden bepaald door de concentratie van het paraproteïne nauwkeurig te volgen, aangezien het percentage plasmacellen en de paraproteïne concentratie aan elkaar gecorreleerd zijn.

Vooralsnog is het multipel myeloom moeilijk te behandelen. Een genezende behandeling is tot op heden maar beperkt beschikbaar. De gemiddelde levensverwachting voor multipel myeloom patiënten bedraagt 3 tot 4 jaar na diagnose van de ziekte. De behandeling van multipel myeloom bestaat voornamelijk uit chemotherapie waarbij verschillende cytostatica worden gecombineerd. Uit recentelijk uitgevoerde klinische onderzoeken is gebleken dat hoge dosis

chemotherapie en stamceltransplantatie een verbeterde overleving tot gevolg heeft. Bij een allogene stamceltransplantatie wordt gebruik gemaakt van stamcellen die afgenomen zijn van veelal een naaste familielid. Afstoting van de getransplanteerde stamcellen en/of afstoting van de ontvanger-lichaamscellen door de getransplanteerde stamcellen kunnen echter ernstige complicaties opleveren na allogene stamceltransplantatie. Bij autologe stamceltransplantatie wordt dit probleem omzeild door gebruik te maken van lichaamseigen hematopoïetische stamcellen. Deze lichaamseigen stamcellen worden voorafgaand aan de hoge dosis chemotherapie geïsoleerd. Door patiënten te behandelen met een hematopoïetische groeifactor zoals G-CSF, migreren stamcellen vanuit het beenmerg naar de bloedsomloop. Deze stamcellen worden vervolgens geïsoleerd uit het bloed, waarna ze ingevroren worden voor later gebruik. Na de hoge dosis chemotherapie worden de ingevroren stamcellen ontdooid en ingespoten. Deze stamcellen migreren naar het beenmerg alwaar ze zorgen voor de aanmaak van nieuwe bloedcellen. Hoge dosis chemotherapie gevolgd door autologe stamceltherapie is tegenwoordig een veel gebruikte behandeling voor myeloom patiënten. In 37 myeloom patiënten die werden behandeld met intensieve chemotherapie gevolgd door autologe stamceltherapie hebben we zowel voor als na de behandeling de paraproteïnen in het serum bepaald. Opvallend was dat vlak na de behandeling in het bloed van veel patiënten (73%) meerdere nieuwe paraproteïnen werden gevonden (oligoklonale paraproteïnen), welke vaak van een ander type waren dan het originele paraproteïne. Klinisch was er geen verschil tussen de patiënten met zulke oligoklonale serum eiwitten, en patiënten zonder oligoklonale paraproteïnen. Met behulp van een gevoelige moleculaire techniek (allel-specifieke polymerase ketting reactie) hebben we vervolgens aangetoond dat de nieuwe oligoklonale paraproteïnen niet afkomstig waren van kwaadaardige cellen die een switch in paraproteïne-type hadden ondergaan. Een opvallend fenomeen in het ziekteproces van multipel myeloom is dat de kwaadaardige plasmacellen nauwelijks een verhoogde delingsactiviteit hebben. Middels gevoelige technieken hebben wij, en anderen, aangetoond dat er in de bloedsomloop van myeloom patiënten B cellen aanwezig zijn welke (nog) niet volledig zijn uitgerijpt tot het plasmacel-stadium, maar wèl tot de kloon behoren waartoe ook de kwaadaardige plasmacellen in het beenmerg behoren. Het zou kunnen dat deze zogenaamde klonotypische B cellen het delende compartiment van de myeloom kloon vormen. Volgens deze hypothese kunnen de klonotypische B cellen tot de kwaadaardige cellen gerekend worden. Aanvulling van de kwaadaardige plasmacellen zou dan plaatsvinden door het continu delen en uitrijpen van de klonotypische B cellen. Om het gedrag van de klonotypische B cellen te karakteriseren, en in kaart te brengen of deze cellen wezenlijk anders zijn dan niet-klonotypische B cellen, hebben we B cellen uit de bloedsomloop van myeloom patiënten gestimuleerd met verscheidene factoren die deling dan wel uitrijping van B cellen kunnen bevorderen. Onder deze verschillende omstandigheden hebben we de aanwezigheid en hoeveelheid klonotypische B cellen bepaald. Klonotypische B cellen lieten een vergelijkbare delingsactiviteit zijn als niet-klonotypische (normale) B cellen na stimulatie

met CD40 ligand en interleukine-4. In het bloed van patiënten werden klonotypische B cellen aangetoond die immunoglobulinen van een ander isotype maakten dan de kwaadaardige plasmacellen in het beenmerg van deze patiënten. Deze zogenaamde circulerende isotype-varianten vertoonden echter geen verhoogde delingsactiveit vergeleken met normale B cellen. Uitgroei van nieuwe klonotypische isotype-varianten werd niet gevonden na stimulatie. Na stimulatie met factoren die B-cel uitrijping tot plasmacel stimuleren bleven klonotypische B cellen aantoonbaar. De relatieve hoeveelheid klonotypische B cellen bleek echter gedaald, wat suggereert dat deze B cellen minder in staat zijn om te reageren op de uitrijpingssignalen dan normale B cellen. Deze resultaten laten zien dat klonotypische B cellen een normale delingscapaciteit hebben maar een verminderd uitrijpingsvermogen. Deze resultaten spreken de hypothese tegen dat klonotypische B cellen het delende en continu uitrijpende compartiment vormen in multipel myeloom. Op dit moment zijn er nog niet voldoende bewijzen dat klonotypische B cellen een cruciale rol spelen in het multipel myeloom ziekteproces. Zorgvuldige klinische studies waarin met name klonotypische B cellen het doelwit vormen van (nieuwe) medicijnen zoals monoklonale antistoffen of specifieke vaccinaties zijn nog niet geïnitieerd of afgerond. Zulke studies zouden het belang van klonotypische B cellen in multipel myeloom patiënten verder kunnen ophelderen.

Analyse van de DNA volgorde dat codeert voor het immunoglobuline dat geproduceerd wordt door de myeloom cellen wijst uit dat deze cellen een selectie proces hebben ondergaan op basis van reactiviteit met lichaamsvreemde stoffen. Dit proces speelt zich af in zogenaamde kiemcentra van de secundaire lymfoïde weefsels. Deze moleculaire signatuur is ook aanwezig in klonotypische B cellen. Vergelijkbare normale B cellen in de bloedsomloop zijn de geheugen B cellen. Deze gegevens wijzen uit dat de myeloom kloon ontstaan is vanuit een B-cel die een kiemcentrum reactie heeft ondergaan. Recentelijk is aangetoond dat geheugen B cellen in de bloedsomloop zich onderscheiden van andere B cellen door de expressie van het membraan-oppervlakte molecuul CD27. Dit molecuul stelt de geheugen B cellen in staat snel uit te rijpen tot plasmacellen. Aangezien het immunoglobuline dat tot expressie wordt gebracht door klonotypische B cellen sterk lijkt op dat van normale geheugen B cellen hebben we de expressie van CD27 op myeloom plasmacellen en klonotypische B cellen bepaald. CD27-positieve circulerende B cellen werden van CD27-negatieve circulerende B cellen gescheiden en vervolgens werd het percentage klonotypische B cellen in beide populaties bepaald. Het percentage klonotypische B cellen bleek in beide populaties echter heel erg klein te zijn en nauwelijks te verschillen. De expressie van CD27 op de myeloom plasmacellen was zeer heterogeen. Patiënten die een goede klinische respons vertoonden op de behandeling hadden plasmacellen die voornamelijk CD27-positief waren. Patiënten met een slechte, of geen respons op therapie werden gekenmerkt door de aanwezigheid van voornamelijk CD27-negatieve plasmacellen in het beenmerg. Dit werd bevestigd door de CD27-expressie van plasmacellen te bepalen uit patiënten die op basis van een genexpressie profiel een slechte dan wel een

goede klinische prognose hadden. Patiënten uit de slechte prognose groep lieten het laagste CD27-expressie niveau zien, ten opzichte van de groep met een goede prognose. Deze gegevens suggereren dat CD27-expressie van plasmacellen een prognostische waarde zou kunnen hebben. In veel onbehandelde patiënten werden zowel CD27-positieve als CD27-negatieve plasmacellen gevonden. Plasmacellen uit het beenmerg van gezonde personen vertoonden een hoge CD27-expressie. Dit suggereert dat de CD27-positieve plasmacellen in myeloom patiënten gezonde, niet-kwaadaardige plasmacellen zouden zijn. Moleculaire analyse wees echter uit dat zowel CD27-positieve als CD27-negatieve plasmacellen tot de kwaadaardige kloon gerekend kunnen worden. Het is vooralsnog onduidelijk of CD27-negatieve plasmacellen uit de CD27-positieve plasmacellen ontstaan of dat ze onafhankelijk van elkaar bestaan. Onze resultaten wijzen uit dat progressie van de ziekte gepaard gaat met een verlaagde CD27-expressie op de plasmacellen. Dit zou veroorzaakt kunnen worden door hetzij een verminderde expressie van CD27, of door uitgroei van CD27-negatieve plasmacellen. Myeloom cellijnen geïsoleerd uit patiënten met progressieve ziekte vertoonden geen expressie van CD27. Wanneer we CD27 geforceerd tot expressie brachten op twee van deze cellijnen resulteerde dat in een snelle celdood. Opvallend was dat deze cellijnen het ligand voor CD27 (CD70) sterk tot expressie brachten. Ook wanneer we deze cellijnen samenbrachten met een andere cellijn die een sterke expressie van CD27 vertoonde, resulteerde dat in een vergelijkbare snelle celdood. Gebaseerd op deze gegevens hypothetiseren wij dat de signalering via CD27 niet zozeer een schadelijk effect heeft op kwaadaardige plasmacellen, maar dat vooral CD70-signalering in kwaadaardige plasmacellen tot celdood kan leiden. Klonale selectie is een belangrijk kenmerk van alle vormen van kanker, en heeft tot gevolg dat de veranderingen die leiden tot een versterkte groei of verminderde celdood van de kwaadaardige cellen met name hierin tot uitdrukking komen. Een verminderde expressie van CD27 op kwaadaardige plasmacellen zou een verminderde CD70-gemedieerde celdood tot gevolg kunnen hebben en dus bijdragen aan de klonale selectie.

Analyse van kwaadaardige plasmacellen uit het bloed van patiënten met een zogenaamde primaire plasmacel-leukemie wees uit dat deze cellen een hoge CD27-expressie hebben. Plasmacel-leukemie is een agressieve ziekte die sterk gerelateerd is aan het multipel myeloom. Het niveau van CD27-expressie op de kwaadaardige plasmacellen is echter duidelijk anders dan in myeloom patiënten. Ondanks het feit dat plasmacel-leukemie een agressiever ziektebeloop heeft, zijn de plasmacellen CD27-positief, een eigenschap die in myeloom patiënten gunstig genoemd zou kunnen worden. De functie van CD27-expressie op plasmacellen van een plasmacel-leukemie patiënt werd onderzocht door de geïsoleerde plasmacellen van een patiënt te stimuleren met CD70, de natuurlijke ligand van CD27. CD27-triggering had een bescheiden celdood-remmende werking, die gepaard ging met activatie van intracellulaire eiwitten die betrokken zijn bij het tegengaan van celdood. Specifieke remming van deze activatie had tot gevolg dat de kwaadaardige plasmacellen gevoeliger werden voor

dexamethason, een stof die gebruikt wordt in de behandeling van myeloom patiënten en celdood van plasmacellen veroorzaakt. Deze bevindingen geven aan dat CD27-expressie voordelig zou kunnen zijn voor de kwaadaardige plasmacellen in het bloed van plasmacel-leukemie patiënten. Remming van specifieke signaalroutes zou een aantrekkelijke mogelijkheid bieden om deze cellen gevoeliger te maken voor behandeling met bijvoorbeeld dexamethason. Het verschil in expressie en functie van CD27 tussen plasmacellen in multipel myeloom en primaire plasmacelleukemie patiënten suggereert dat verschillende ziekteprocessen van belang zijn. Dit zou een aanwijzing kunnen zijn dat beide ziekten minder aan elkaar gerelateerd zijn dan aanvankelijk werd gedacht.

Remming van intracellulaire signaalroutes die betrokken zijn bij resistentie-vorming voor chemotherapeutica en apoptose vormen potentiële doelwitten voor het ontwikkelen van nieuwe medicijnen en behandelingen. De recentelijk ontwikkelde microarray-methoden maken het mogelijk om de expressie van een groot aantal genen tegelijk te bepalen en daarmee een beeld te geven welke signaalroutes gedereguleerd zijn in kwaadaardige cellen. Vergelijkbare technische ontwikkelingen op het gebied van de eiwitchemie ('proteomics') zullen in de nabije toekomst mogelijk bijdragen aan de opheldering van veranderingen die optreden op eiwit-niveau. Hiermee zal wellicht de identificatie van specifieke ziekteprocessen aanzienlijk vergemakkelijkt worden. Daarbij is het op deze wijze ook mogelijk patiënten te selecteren die in aanmerking komen voor behandeling met medicijnen die zulke signaalroutes als doelwit hebben. Ondanks het feit dat multipel myeloom patiënten veel overeenkomstige karakteristieken hebben zijn er ook belangrijke verschillen tussen individuele patiënten. Sommige van deze heterogene karakteristieken hebben voorspellende waarde voor de respons op therapie en de uiteindelijke overleving van de patiënt. Het is van groot belang de biologische functie en oorzaak van zulke eigenschappen te identificeren omdat deze belangrijke aanwijzigingen kunnen geven omtrent het ziekteproces. De identificatie van (klinische) subtypes van het multipel myeloom zullen uiteindelijk de ontwikkeling van 'op maat gemaakte' behandelingen voor individuele patiënten of patiëntengroepen mogelijk maken. Het in kaart brengen en waarderen van de heterogeniteit van het multipel myeloom is daarom van cruciaal belang voor de ontwikkeling van nieuwe behandelingen en zal hopelijk resulteren in verbeterde overlevingskansen voor multipel myeloom patiënten.

LIST OF ABBREVIATIONS

AET	:	2-aminoethylisothiouronium-hydrobromide
AP-1	:	activator protein-1
APC	:	allophycocyanin
ASCT	:	autologous stem cell transplantation
ASO	:	allele specific oligonucleotide
BC	:	B-cell
BM	:	bone marrow
bp	:	basepair
BPC	:	bone marrow plasma cell
BSA	:	bovine serum albumine
CALLA	:	common acute leukaemia antigen
CD	:	cluster of differentiation
CDR	:	complementarity determining region
CFSE	:	carboxyfluorescein diacetate succinimidyl ester
CFU-GM	:	colony forming unit granulocyte macrophage
CGH	:	comparative genomic hybridisation
CH	:	immunoglobulin heavy chain constant region gene
CI	:	confidence interval
CR	:	complete remission
CSR	:	class switch recombination
Cα	:	constant region alpha
Cδ	:	constant region delta
Cε	:	constant region epsilon
Cγ	:	constant region gamma
Cμ	:	constant region mu
D	:	diversity gene segment
DEPC	:	diethylpyrocarbonate
ECL	:	enhanced chemoluminescence
EDAP	:	etoposide dexamethasone ara-C cisplatin
EFS	:	event free survival
EMSA	:	electrophoretic mobility shift assay
ENS	:	enhanced normalisation substraction
ERK1/2	:	extracellular regulated kinase 1/2
FACS	:	fluorescence activated cell sorter
FCS	:	fetal calf serum
FISH	:	fluorescence in situ hybridisation
FITC	:	fluorescein isothiocyanate
FR	:	framework
FSC	:	forward scatter
GM-CSF	:	granulocyte macrophage colony stimulating factor
Gy	:	Gray
HBSS	:	Hank's balanced salt solution
HDM	:	high dose melphalan
HGF	:	hematopoietic growth factor
HLA	:	human leucocyte antigen
IFN	:	interferon
Ig	:	immunoglobulin
IgH	:	immunoglobulin heavy chain
IgL	:	immunoglobulin light chain
IL	:	interleukin

JH	:	immunoglobulin heavy chain joining gene segment
JNK	:	Janus N-terminal kinase
L	:	ligand
LCA	:	leucocyte common antigen
log	:	logarithmic
M	:	monoclonal
MAPK	:	mitogen activated protein kinase
MFI	:	mean fluorescence intensity
MGUS	:	monoclonal gammopathy of undetermined significance
MHC	:	major histocompatibility complex
MM	:	multiple myeloma
MNC	:	mononuclear cell
NCAM	:	neural cell adhesion molecule
NCS	:	newborn calf serum
NF-κB	:	nuclear factor-kappa B
NOD/SCID	:	non-obese diabetic/severe combined immunodeficient
OM	:	oncostatin M
OS	:	overall survival
PB	:	peripheral blood
PBS	:	phosphate buffered saline
PBSC	:	peripheral blood stem cell
PBSCT	:	peripheral blood stem cell transplantation
PC	:	plasma cell
PCL	:	plasma cell leukaemia
PCR	:	polymerase chain reaction
PE	:	phycoerythrin
PerCP	:	peridine chlorophyll protein
PR	:	partial remission
RAG	:	recombination activating gene
RE	:	responsive element
RT	:	reverse transcription
SAPK	:	stress activated protein kinase
SB203580	:	4-4-fluorophenyl-2-4-methylsulfinylphenyl-5-4-pyridyl 1H-imidazole
SDS	:	sodium dodecyl sulphate
SHM	:	somatic hypermutation
SRBC	:	sheep red blood cell
SSC	:	side scatter
SSC	:	standard saline citrate
t	:	translocation
TBE	:	tris borate EDTA
TdT	:	terminal deoxynucleotidyl transferase
TNF	:	tumour necrosis factor
TNFR	:	tumour necrosis factor receptor
TPC	:	tonsillar plasma cell
U0126	:	1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)-butadiene
UV	:	ultraviolet
VAD	:	vincristine adriamycine dexamethasone
VH	:	immunoglobulin heavy chain variable gene segment
VL	:	immunoglobulin light chain variable gene segment
X-gal	:	5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside

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Jeroen

CURRICULUM VITAE

Jeroen Guikema werd geboren op 3 februari 1974 in Hoogezand-Sappemeer, waar hij zowel de kleuterschool als het basisonderwijs met succes doorliep. Daarna volgde het St. Maartenscollege in Haren (atheneum B). In 1992 besloot hij te breken met de familietraditie, en dus geen kapper te worden. In plaats daarvan ging hij Biologie studeren aan de Rijksuniversiteit Groningen, waarbij hij na anderhalf jaar ganzen en eenden observeren, planten determineren, slakken tellen en grassprieten meten, koos voor de afstudeerrichting Medische Biologie. Onderzoekstages werden uitgevoerd bij de toenmalige vakgroep Histologie & Celbiologie van de faculteit der Medische Wetenschappen aan de Rijksuniversiteit Groningen, waar onder leiding van dr. Nico Bos reeds de basis werd gelegd voor dit proefschrift. Daarna werd 6 maanden onderzoek uitgevoerd op de afdeling Hematologie & Immunologie van de Vrije Universiteit Brussel in België, onder leiding van dr. Marleen Bakkus. De afstudeerbul Medische Biologie werd in augustus 1997 cum laude gehaald.

Daags na het afstudeerfeest werd als AIO aangevangen met het onderzoeksproject 'de rol van de klonotypische B cel in multiple myeloma' op het laboratorium van de Histologie & Celbiologie, faculteit der Medische Wetenschappen, RuG, onder directe begeleiding van dr. Nico Bos, in samenwerking met prof. dr. Edo Vellenga van de afdeling Hematologie van het Academisch Ziekenhuis Groningen. Het AIO-project heeft geresulteerd in dit proefschrift.

Jeroen Guikema is momenteel werkzaam als post-doc bij de afdeling Pathologie & Laboratoriumgeneeskunde van het Academisch Ziekenhuis Groningen, waar hij in de onderzoeksgroep van dr. Ed Schuuring en prof.dr. Philip Kluin onderzoek doet naar de rol van immunoglobuline klasse switch recombinatie in de vorming van B-cel lymfomen.

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